

PARALLEL MECHANISMS OF STEROID SIGNALING AND MALE
ALTERNATIVE REPRODUCTIVE TACTICS IN THE MIDSHIPMAN FISH,
PORICHTHYS NOTATUS

A Dissertation

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by

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The consequences of corticosteroid activity are as diverse as they are necessary. Steroid hormones and their receptors have co-evolved to influence a multitude of physiological and behavioral parameters via discrete modifications in steroid signaling pathways. Here, I investigated the presence and relative abundance of receptors and steroidogenic enzymes in the corticosteroid (glucocorticoids and mineralocorticoids) and androgen signaling pathways during the breeding season in a teleost fish, the plainfin midshipman (*Porichthys notatus*), that has two male reproductive morphs with divergent reproductive tactics. Territorial (type I) males acoustically court females, whereas sneaker (type II) males do not court females but rather steal fertilizations from type I males. I first cloned and sequenced the midshipman genes for glucocorticoid (GR) and mineralocorticoid (MR) receptors and the enzymes 11 β -hydroxysteroid dehydrogenase (11 β HSD) and 11 β -hydroxylase (11 β H) that are involved in corticosteroid and androgen synthesis. A prior study cloned and sequenced the midshipman androgen receptor (AR). Absolute quantitative PCR then determined the mRNA expression profiles for GR, MR, AR, 11 β HSD and 11 β H in each midshipman male morph. In general, type II males showed the highest mRNA expression levels for all genes throughout the central nervous system. Type I males, however, had higher mRNA levels for all genes in the vocal muscle, and for 11 β HSD and 11 β H in the testis. Together, the expression patterns showed that mRNA

abundance for the enzymes and receptors mediating androgen and glucocorticoid signaling pathways are distinct in type I and II male midshipman fish, reflecting their equally distinct vocal, spawning and endocrine phenotypes. A separate study addressed the specificity of the ligand binding domain of corticosteroid receptors from teleost fish (including midshipman) and mammals. The results indicated that a duplicated GR in teleosts exhibited high sequence identity to the mammalian GR, but responded like the mouse MR to steroid ligands. A phylogenetic analysis then showed that the duplicated fish GR secondarily evolved after a more ancestral duplication event that led to the initial divergence of two corticosteroid receptors in vertebrates that were activated by either glucocorticoids alone (GR) or both glucocorticoids and mineralocorticoids (MR).

BIOGRAPHICAL SKETCH

Adam was born the son of Paul Arterbery and Rinda Starr-Alsip, and brother to Andrew Arterbery in Stillwater, Oklahoma on May 19, 1981. During his early childhood he spent every moment he could, following step-for-step behind his late Grandfather on the Starr family farm. He graduated from Stillwater High School in the summer of 2000 and began his freshman year of college at the University of Denver the following fall. While in college, Adam began his scientific research career in the laboratories of Dr. Robert Does and Dr. Catherine Kunst. He completed college in 2004 with a B.S. in Biology, a B.S. in psychology and a chemistry minor. He then moved to Ithaca, NY to pursue his PhD at Cornell University in the Department of Neurobiology and Behavior in the Lab of Dr. Andrew Bass. Over the years, he has continued to develop as a dedicated musician and gained a strong passion for his pursuit in the martial arts of Brazilian Jiu Jitsu and Wushu. His passion for science grew from a young age as he followed his Grandfather around the family farm. From the Midwestern plains to the Rocky mountains, and the mountains to the Finger Lakes, his journey continues following an old Native American Proverb: “If we often wonder, the gift of knowledge will come.”

Dedicated to my Grandfathers John L. Starr and William J. Arterbery, my brother
Andy, my mother Rinda, and my father Paul, the Starr Family, the Arterbery family,
and the Alsip family.

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LIST OF ABBREVIATIONS

CR	Corticosteroid Receptor
GR	Glucocorticoid Receptor
MR	Mineralocorticoid Receptor
AR	Androgen Receptor
11 β HSD	11 beta-hydroxysteroid dehydrogenase
11 β H	11 beta-hydroxylase
S	11-Deoxycortisol
F	Cortisone
M	Cortisol
11KT	11-ketotestosterone
VM	Vocal muscle
VH-SC	Vocal Hindbrain-Spinal Cord
OB	Olfactory bulb
Tel	Telencephalon
POA	Pre-optic area
Mid	Midbrain
Di	Diencephalon
Cbl	Cerebellum

CHAPTER 1

INTRODUCTION: STEROID HORMONES, RECEPTORS, AND ENZYMES

The steroid-related control over reproduction and stress-response are ancient and critical regulatory processes among vertebrates (1). Steroid receptors are members of the nuclear receptor superfamily of proteins that are found in both protostomes (including insects) and deuterostomes (including vertebrates) (2, 3). The available evidence now suggests that a series of genomic duplications within deuterostomes may have resulted in the diverse family of steroid receptors that have contributed, in part, to the evolution of the complex signaling mechanisms between the endocrine and nervous systems of vertebrates.

Three important “players” can have important consequences for the proper functioning of steroid-dependent regulatory mechanisms: the steroid hormone or ligand, the steroid receptor and steroidogenic enzymes that lead to the conversion of one steroid to another. Genetically-based dysfunction in the function of any one of the above elements can lead to a wide range of disease-related states in humans (e. g., depression, schizophrenia, cancer) (6, 7). Thus, one major question regarding steroid signaling mechanisms is: How do individual differences in physiology and behavior relate to genomic differences in the expression of steroid receptors and steroidogenic enzymes that underlie steroid hormone synthesis? Chapters 1 and 2 of this thesis investigate a genetic basis for glucocorticoid and androgen signaling mechanisms in a species of teleost fish, the plainfin midshipman (*Porichthys notatus*), that has two male reproductive morphs that exhibit widely divergent alternative reproductive tactics. Male alternative reproductive tactics are widespread among teleost fish and occur among all major groups of tetrapods (including primates), and offer a unique opportunity to investigate the molecular basis of divergent steroid signaling

mechanisms. Vertebrates with alternative behavioral phenotypes provide naturally-occurring models for identifying steroid-dependent mechanisms that promote the successful transitioning from disease to adaptive behavioral and physiological states.

Chapter 3 of this thesis explores the evolution of the ligand specificity of steroid receptors. The synthetic pathways leading to diverse steroid hormones among vertebrates have been extensively studied in all of the major vertebrate groups (5). However, the evolutionary events that have led to the ligand specificity of steroid receptors are less clear and have just begun to be studied. Chapter 3 focuses on corticosteroid signaling mechanisms. The presence of mineralocorticoid (MR)-like and duplicate glucocorticoid (GR)-like receptors in teleost fish has been an area of much investigation because of remaining uncertainties in how they compare functionally to the MR and GR of tetrapods. A minimal promoter based functional assay is used to compare the specificity of the ligand binding domain (LBD) of corticosteroid receptors from two distantly related groups of teleost fish, cichlids and midshipman, to the MR and GR of a mammal (mouse). A phylogenetic analysis then places the results into the context of the evolutionary relationships of MRs and GRs based on a detailed analysis of their amino acid sequences. The results suggest that specific residue shifts within the protein sequence of corticosteroid receptors may have led to divergent receptor-ligand specificity that evolved multiple times over the course of vertebrate evolution. Together, the results begin to clarify the evolutionary relationships between steroids, the enzymes that make them, and the receptors that bind them.

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CHAPTER 2

CORTICOSTEROID RECEPTOR EXPRESSION IN A TELEOST FISH THAT DISPLAYS ALTERNATIVE MALE REPRODUCTIVE TACTICS

Abstract

Corticosteroid signaling mechanisms mediate a wide range of adaptive physiological responses, including those essential to reproduction. Here, we investigated the presence and relative abundance of corticosteroid receptors during the breeding season in the plainfin midshipman fish (*Porichthys notatus*), a species that has two male reproductive morphs. Only type I “singing” males acoustically court females and aggressively defend a nest site, whereas type II “sneaker” males steal fertilizations from nesting type I males. Cloning and sequencing first identified glucocorticoid (GR) and mineralocorticoid (MR) receptors in midshipman that exhibited high sequence identity with other vertebrate GRs and MRs. Absolute quantitative real time PCR then revealed higher levels of GR in the central nervous system (CNS) of type II males than type I males and females, while GR levels in the sound-producing, vocal muscle and the liver were higher in type I males than type II males and females. MR expression was also greater in the CNS of type II males than type I males or females, but the differences were more modest in magnitude. Lastly, plasma levels of cortisol, the main glucocorticoid in teleosts, were two to three fold greater in type II males compared to type I males. Together, the results suggest a link between corticosteroid regulation and physiological and behavioral variation in a teleost fish that displays male alternative reproductive tactics.

Introduction

Among vertebrates, the hormonal regulation of responses to environmental stressors (both biotic and abiotic) is ancient and includes critical regulatory processes mediated by corticosteroid signaling mechanisms (1, 2). Steroid receptors are ligand-activated transcription factors that mediate the actions of steroid hormones, including corticosteroids, on a diversity of functions ranging from sexual differentiation and adult reproductive behavior to immune responses, sodium reabsorption, and anti-inflammatory processes (3, 4). Steroid receptors, including those targeted by corticosteroids, are a subfamily of the greater superfamily of nuclear receptors that are composed of several domains important for transcriptional activation (5). Vertebrates have two main classes of corticosteroid receptors, glucocorticoid (GR) and mineralocorticoid (MR) (3). While both receptor classes have long been known for tetrapods, more recent studies have identified a single corticosteroid receptor in jawless fish (lamprey and hagfish), but distinct GR and MR in cartilaginous and teleost fishes (2, 6).

Teleosts show a wide range of neuroendocrine phenotypes reflecting, in part, an equally divergent range of reproductive tactics (7, 8). Here, we investigated the presence and abundance of corticosteroid receptors in the plainfin midshipman (*Porichthys notatus*) that has two male morphs that follow either a displaying/courting or a sneak-satellite spawning tactic (8, 9). Territorial, type I males build and guard nests (females offer no parental care) and acoustically court females with a long duration courtship “hum” (mins to > 1 h) involving the rapid contraction (~100 Hz at 16°C) of paired vocal muscles attached to the walls of the gas-filled swimbladder that are considered among the fastest known vertebrate muscles (10-12). Type I males also make shorter “growls” (sec-mins) and very brief “grunts” (msec) during intraspecific,

aggressive encounters (11, 13). Sneak/ satellite spawning, type II males steal fertilizations from nesting type I males, are about 50% smaller than type I males and have a nine fold greater testes/ body mass ratio than type I males (11). Type II males (and females) are known so far to only produce grunts in non-spawning contexts (11).

The divergent vocal repertoires between the two male morphs are paralleled by a large suite of neural and non-neural mechanisms (7, 9, 14). For example, the vocal muscles of type I males have several traits that are greater in magnitude than those of type II sneaker males (and females), ranging from overall mass to fiber number and diameter, the abundance of mitochondria in the sarcoplasm, and the ultrastructural dimensions of myofibrils (10, 15). The divergence in vocal behavior and muscle traits is indicative of an even larger number of morph-specific traits in the vocal control network that determines the temporal properties of natural vocalizations (14). In sum, the type I male morph exhibits a large suite of morphological and physiological traits mainly adapted to the performance of a metabolically demanding behavior, namely advertisement calling that may last for more than one hour (see above - 11, 13, 16).

Corticosteroids, as well as androgens and estrogen, have been proposed to influence the development and adult maintenance of alternative male morphs in midshipman fish and other teleosts (17-19). Cortisol, the predominant corticosteroid in teleosts (4), can modulate the activity pattern of a hindbrain-spinal vocal pattern generator that directly establishes the temporal features of midshipman vocalizations (14). Intramuscular, dorsal trunk injections of cortisol in type I males increases the duration of the vocal motor, occipital nerve volley that reflects the activity of the hindbrain-spinal pattern generator, but suppresses the duration of the motor volley in type II males (19).

Male morph-specific patterns of cortisol action on vocal as well as non-vocal behaviors might depend, in part, on differential patterns of expression of corticosteroid

receptors. Hence, the aim of the present study was to more directly test the hypothesis that both glucocorticoid and mineralocorticoid receptors show male morph-specific patterns of expression in the central nervous system and peripheral tissues in midshipman fish. We also tested the corollary that plasma levels of cortisol would differ between the two male morphs. A prior study of only type I males and females showed that cortisol levels were significantly higher in females, but only during the non-breeding season when fish are collected from deep offshore sites (20).

Materials and Methods

Animals and Blood Sampling

Midshipman fish were collected from field-nest sites in northern California during the summer breeding season of May-August (see 9), shipped to Cornell University within 72h and maintained in seawater tanks until sacrificed within 24h for tissue collection. The size of the testis and vocal muscle provided characters to easily identify each sex and male morph upon visual inspection (9). This study included all three adult reproductive morphs: type I males (20.6 – 25.1 grams, 12-13.5 cm), type II males (3.94-5.88 grams, 8-8.5 cm) and females (11.12-17.5 grams, 10-11.5 cm). Tissue sampling and blood collection were carried out following deep anesthetization (0.025% benzocaine; Sigma, St. Louis, MO). All experimental protocols were approved by the Cornell University Institutional Animal Care and Use Committee. Blood was obtained for cortisol assays by cardiac puncture within three - four hours after collection from nest sites in a subset of type I (n=5) and type II males (n=6) (20, 21); a previous study compared females to type I males (20). Blood plasma was collected after centrifugation and frozen for later analysis by radioimmunoassay (RIA) at the Diagnostic Laboratory, New York State College of Veterinary Medicine at Cornell, Ithaca, NY. Like most studies in teleosts (reviewed in 22), aldosterone was

not detected in RIAs for blood samples collected as above from three additional type I males and hence further assays were not done, in part, because of limited availability.

Cloning, Sequencing, and Alignment

RNA was isolated and pooled from type I male liver (n=3) from the breeding season using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. PCR on cDNA from all tissues were conducted using degenerate primers as follows. **Glucocorticoid receptor**: forward primer (PACRFRK): 5'-CCN-GCN-TGY-CGN-TTY-CGN-AAR-TG (T_m=62.0°C); reverse primer (PLLFHQK): 5'-YTT-TTG-RTG-RAA-NAG-NAG-NGG (T_m=52.4°C). **Mineralocorticoid receptor**: forward primer (PYLTSPV): 5'-CCV-TAY-CTG-MCS-CCS-TCC-RTC (T_m=62.3°C); reverse primer (DLVLGMR): 5'-CCK-CAT-SCC-CAS-CAC-CAG-RTC (T_m=62.8°C). The primers were designed to amplify a highly conserved region based on an alignment of vertebrate corticosteroid receptors (see Figs. 2.1, 2.2). **Beta actin** primers were designed from *Acanthopagrus schlegelii* (Genbank Accession No. AY491380); forward primer (GDGWTHT): 5' GGT-GAT-GGT-GTG-ACC-CAC-ACA-GTG (T_m=61.9°C); reverse primer (KYSVWIG): 5' TTT-ATG-AGA-CAG-ACC-TAG-CCT (T_m=54.3°C).

Amplification was performed with Taq polymerase (Epicentre Biotechnologies, Madison, WI) using the following cycles: Stage 1 – 94°C for 2 min (1 cycle), Stage 2 – 94°C for 1 min, 55°C for 1 minute, 72°C for 3 min (35 cycles), and Stage 3 – 72°C extension for 10 minutes (1 cycle). Products were run on a 1% agarose gel and verified to be the approximate expected size: a 906 bp product for GR,

Figure 2.1: An alignment of the ligand binding domain (LBD) for vertebrate glucocorticoid receptors (GR) reveals high sequence identity between multiple vertebrate species. From the 5' end of the alignment, the areas highlighted are the final residues that code for the DNA binding domain (DBD), the species-specific hinge region, and the LBD for which activational domains (AF2) and helices (h1-12) are noted. Forward (5') and reverse (3') primers are indicated for both cloning and qPCR procedures.

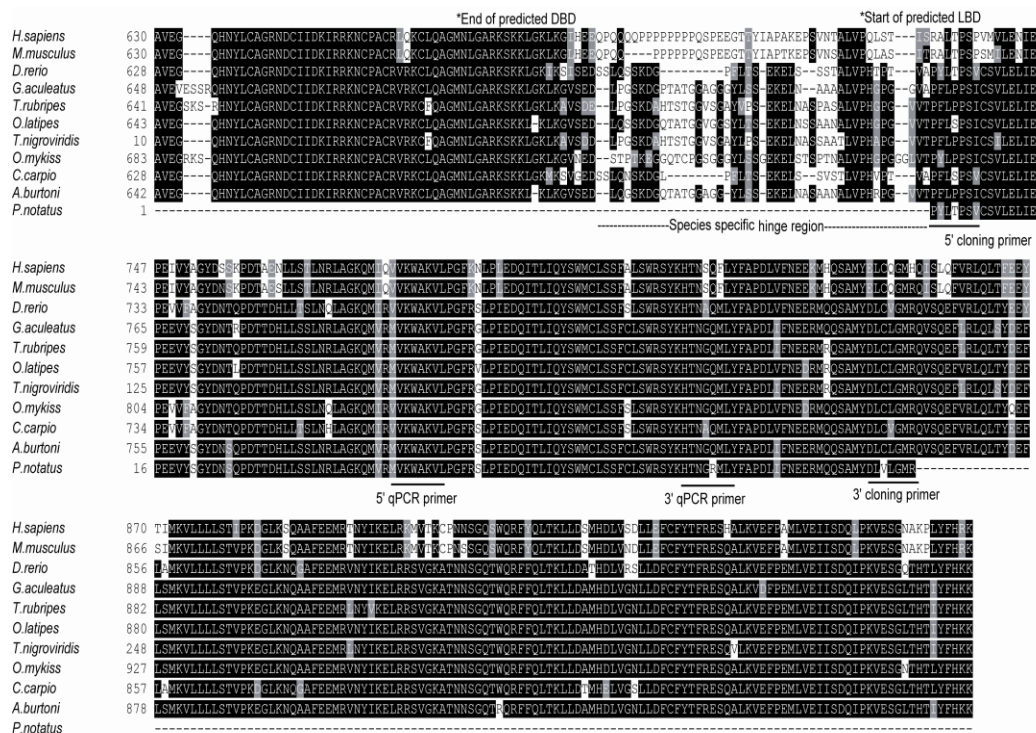


Figure 2.2: An alignment of vertebrate mineralocorticoid receptors. The sequences begin at the 3' end of the DNA binding domain (DBD), include the species-specific hinge region, and the full length ligand binding domain (LBD). The areas highlighted are the final residues that code for the DBD, the species-specific hinge region, and the LBD. Forward (5') and reverse (3') primers are indicated for both cloning and qPCR procedures.

a 366 bp product for MR, and a 564 bp product for beta actin. The primers contained restriction sites on the 5 prime ends to increase the efficiency of ligation into the Bluescript KS (+) plasmid, and the products were subcloned using DH5 α competent cells and plated. Twenty individual colonies were grown in liquid culture, mini-prepped (Qiagen, Germantown, MD), and sequenced at the Cornell University Life Sciences Core Laboratory Center using T3 and T7 primers. The resulting sequences were subject to a BLAST analysis (NCBI) to verify identity and for alignment with other vertebrate sequences (CLustalW).

Sequences for both alignments included a broad range of vertebrates, as follows. **Glucocorticoid receptor:** *Homo sapiens* (GenBank Accession No. AY436590), *Mus musculus* (GenBank Accession No. nm_008173), *Danio rerio* (Genbank accession number NM_001020711.2), *Cyprinus carpio* GR1b (GenBank Accession No. AM697886), *Cyprinus carpio* GR2 (GenBank Accession No. AM183668), *Astatotilapia burtoni* isoform 1 (GenBank Accession No. AF263738), *Gasterosteus aculeatus* GR1 (Ensembl Identifier: ENSGACG000000018209), *Takifugu rubripes* GR2 (Ensembl Identifier: ENSTRUG000000007443), *Oryzias latipes* GR2 (Ensembl Identifier: ENSORLG000000006022), *Tetradon nigroviridis* GR1 (Ensembl Identifier: ENSTNIG000000008946), *Oncorhynchus mykiss* GR2 (GenBank Accession No. NM_001124482), *Oncorhynchus mykiss* GR1 (GenBank Accession No. NM_001124730), *Salmo trutta* (GenBank Accession No. AY863149), *Paralichthys olivaceus* (GenBank Accession No. AB013444), *Dicentrarchus labrax* (GenBank Accession No. AY549305), *Cyprinus carpio* GR1a (GenBank Accession No. AJ879149), *Astatotilapia burtoni* isoform 2b (GenBank Accession No. AF263740). **Mineralocorticoid receptor:** *Homo sapiens* (GenBank Accession No. nm_000901.3), *Mus musculus* (GenBank Accession No. nm_001083906.1), *Danio rerio* (GenBank Accession No. nm_001100403.1), *Gasterosteus aculeatus* (Ensembl Identifier:

ENSGACG000000017193), *Takifugu rubripes* (Ensembl Identifier: ENSTRUG0000000014871), *Oryzias latipes* (Ensembl Identifier: ENSORLG000000007530), *Tetradodon nigroviridis* (Ensembl Identifier: ENSTNIG000000017732), *Oncorhynchus mykiss* (GenBank Accession No. NM_001124483.1), *Cyprinus carpio* (GenBank Accession No. AJ783704), *Astatotilapia burtoni* (GenBank Accession No. AF263741). A second isoform, GR2a, for *A. burtoni* was not included in the alignment because it shows a 100% sequence identity with the partial sequence of *A. burtoni* GR2b used in the analysis (see Greenwood et al., 2003).

Absolute-Quantitative Real Time PCR (qPCR)

The qPCR analysis was based on the analysis of tissues from individual fish (n=4-5 per morph/sex). RNA was isolated using Trizol (Invitrogen, Carlsbad, CA), treated twice with DNaseI (Invitrogen, Carlsbad, CA) to remove genomic DNA contamination, and reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. As in previous biochemical and qPCR studies in midshipman (23, 24), the CNS was divided into three main regions for quantitative analysis: forebrain including olfactory bulb (Ob), telencephalon (Tel) and preoptic area (POA); a large middle region including midbrain tectum and tegmentum (Mid), diencephalon (Di) and cerebellum (cbl); and the remaining hindbrain-rostral spinal cord region (VH-SC) that houses the vocal pattern generator circuit (Bass et al., 1994). Samples were also taken from liver, testis, and vocal muscle (VM). Female gonad was not investigated due to limited availability and a major focus here being on divergence between the two male morphs.

Absolute qPCR was conducted on cDNA from all tissues using gene specific primer pairs that were chosen based on the following criteria: 1) amplify a product

about 150 nucleotides in length, 2) isolate a single product with no dimer pairs, and 3) have a standard efficiency (R^2) of no less than 98%. The following primers were designed from the nucleotide sequence of the midshipman genes. **Glucocorticoid receptor**: the forward primer 5'-GCT-GCA-GTG-CTC-GTG-GCT-TT ($T_m=61.3^\circ\text{C}$) and reverse primer 5'-GCA-TCT-GCT-CGC-ACT-GGT-CA ($T_m=60.3^\circ\text{C}$) were chosen as the primer pair ($R^2=0.984$). **Mineralocorticoid receptor**: the forward primer 5'-GCA-TGG-TGA-AAT-GGG-CCA-AAG-TAC-TTC ($T_m=60.3^\circ\text{C}$) and the reverse primer 5'-AAG-TAG-AGC-ATC-CGT-CC ($T_m=60.6^\circ\text{C}$) were chosen as the primer pair ($R^2=0.981$). **Beta actin**: the forward primer 5'-CCA-CGC-CAT-CCT-GCG-TCT ($T_m=60.7^\circ\text{C}$) and the reverse primer 5'-GCT-CGA-AGT-CCA-GGG-CAA-CA ($T_m=60.1^\circ\text{C}$) were chosen as the primer pair ($R^2=0.987$). All real time reactions were run in triplicate along with no template controls and contained the following: 10 μl of 2X Power SYBR Green PCR Master Mix (Applied Biosystems), 2 μl of forward and reverse primer at a concentration of 100 nM, 4 μl of H_2O , and 2 μl of the appropriate cDNA. Reactions were run on an Applied Biosystems 7900 HT Sequence Detection System at the Cornell University Life Sciences Core Laboratory Center under the default manufacturer's conditions (SDS 2.1 software) using 60°C as a melting temperature.

Gene copy number was determined for each tissue sampled from each individual using standard curve analysis for all gene primer sets, including housekeeping genes. The standards covered a linear range of 5×10^6 to 100 copy/ μl . Briefly, the raw C_t values were converted to copy number with the standard curve produced using the SDS 2.1 (Applied Biosystems). Each target gene copy number was normalized using the beta actin (βA) copy number (n) from the same tissue sample (e.g., forebrain GR n / forebrain $\beta\text{A}n$). Beta actin was chosen, in part, because it had been previously used in mRNA expression studies of midshipman fish (23). Initial

cloning also investigated the use of glyceraldehyde 3-phosphate dehydrogenase (GADPH) as a reference gene, but GADPH primers did not perform consistently. By contrast, the β A gene-specific primers showed excellent qPCR efficiency ($R^2=0.99$, data not shown), and variation in expression of β A was less than 5% between morphs within a given tissue. However, since variation in β A expression was much greater than 15% between tissues, we did not statistically compare expression between peripheral and central tissues. We recognize that β A may yet be regulated in a manner not apparent in the comparisons made for the current study of midshipman. A study of the cichlid *A. burtoni* showed that β A expression differed between reproductive (R) and non-reproductive (NR) males (25), although R and NR males are not comparable to type I and II midshipman, both of whom are reproductively active (Brantley and Bass, 1994).

Statistics

Normalized mRNA values and plasma cortisol levels were compared between morphs for each tissue sampled. One-way ANOVA and post-hoc Tukey-HSD tests were performed on all data sets using JMP 7.0 software. Welch-ANOVA tests were performed for each statistical comparison where unequal variance may influence the analysis.

Results

Cloning and Sequencing

In order to examine whether corticosteroids and their receptors play a role in the alternative reproductive tactics of the midshipman male morphs, we first designed primers that could amplify portions of the midshipman GR and MR genes. The resulting PCR products were subcloned and 20 independent clones of GR and MR

were sequenced. For both GR and MR, the sequencing of multiple clones revealed that only one gene was amplified by each of the degenerate primer pairs, and gene-specific primers also revealed a single product as expected. The translated protein sequence can be viewed on Genbank on the NCBI webserver (Genbank Accession No. EF092836 for GR and EU926160 for MR).

We note that the reverse primer designed for cloning of the midshipman GR is based upon an amino acid sequence that is divergent between the two GR genes in the cichlid *A. burtoni* (GR1 final coding residue is an arginine – R, and GR2b final coding residue is a lysine – K; see GR alignment in Fig. 2.1). The coding nucleotides for these amino acids in the GR sequences of *A. burtoni* differ by one nucleotide in the middle position (GR1: lysine = aaa, GR2b: arginine = aga), and they are both amides (see Fig. 2.1). Thus, we realize that if this amino acid is divergent between potentially duplicated midshipman GR genes, it may not be surprising that only one receptor was amplified with this primer. This variation is, however, not consistent across teleosts; this amino acid is not divergent between the two GR genes in the common carp, *C. carpio* (see Fig. 2.1).

Comparisons of the midshipman receptors with other vertebrate GRs (alignment in Fig. 2.1 and sequence identity in Table 2.1) and MRs (alignment in Fig. 2.2 and sequence identity in Table 2.2) revealed high sequence identity. Steroid receptors are composed of several domains important for transcriptional activation: DNA binding domain (DBD-C domain), ligand binding and dimerization domain (LBD-E domain), N-terminal (A/B-domain), hinge region (D-domain), and C-terminal (F-domain) (5). The aligned sequences in the GR included the entire LBD domain, with its predicted binding residues and a portion of the DBD domain sequence. The midshipman GR was most similar to the cichlid GR2 (Table 1.1) that has a nine amino

Table 2.2: Sequence identity chart for all sequences used in the MR alignment.

	<i>H.sapiens</i>	<i>M.musculus</i>	<i>D.erio</i>	<i>G.aculeatus</i>	<i>T.rubripes</i>	<i>O.latipes</i>	<i>T.nigroviridis</i>	<i>O.mykiss</i>	<i>C.carpio</i>	<i>A.burtoni</i>	<i>P.notatus</i>
<i>H.sapiens</i>	100	95	74	69	67	70	68	69	73	68	74
<i>M.musculus</i>	95	100	74	68	68	70	69	71	73	71	75
<i>D.erio</i>	74	74	100	84	83	85	84	88	95	85	88
<i>G.aculeatus</i>	69	68	84	100	91	92	92	87	84	94	93
<i>T.rubripes</i>	67	68	83	91	100	91	95	87	82	92	93
<i>O.latipes</i>	70	70	85	92	91	100	92	89	87	96	90
<i>T.nigroviridis</i>	68	69	84	92	95	92	100	85	84	93	92
<i>O.mykiss</i>	69	71	88	87	87	89	85	100	86	88	87
<i>C.carpio</i>	73	73	95	84	82	87	84	86	100	83	86
<i>A.burtoni</i>	68	71	85	94	92	96	93	88	83	100	95
<i>P.notatus</i>	74	75	88	93	93	90	92	87	86	95	100

acid insert in the DBD domain (Fig. 2.1). The midshipman GR and MR shared 52% sequence identity in the overlapping sequences of the DBD/ LBD domains.

Absolute qPCR

We hypothesized that the expression of GR and MR in the type I and type II males may differ from each other and reflect their different reproductive tactics. Indeed, robust and significant differences were found between the two male morphs, as well as between both male morphs and females, for both GR and MR in central and peripheral tissues. Welch-ANOVA verified both morph and gene specific differences for both the GR and MR ($p < 0.0001$). The results for GR and MR are presented in graphical format in Figure 2.3, and in numerical detail in Tables 2.3 and 2.4.

For GR, type II males showed significant expression above that of both other morphs in forebrain and the vocal hindbrain-spinal cord (indicated by double asterisks in Fig. 2.3), but only above females in the middle CNS region containing midbrain, diencephalon, and cerebellum (single asterisk in Fig. 2.3) (one way ANOVA, $p \leq 0.013$). Type I male forebrain GR levels were also significantly greater than those for females, ($p = 0.0001$). In the vocal muscle and liver, type I males showed significantly greater levels of GR expression above type II males and females ($p \leq 0.014$). By contrast, there were no differences in levels of expression between the male morphs for the testis ($p = 0.645$).

For MR, type II males again showed higher levels than type I males and females in the vocal hindbrain-spinal cord ($p = 0.002$), but only above type I males in the forebrain ($p = 0.033$). There were no significant differences across all morphs in the middle CNS region. In the periphery, type II males exhibited significantly higher levels of MR over both type I males and females in the vocal muscle alone ($p = 0.002$).

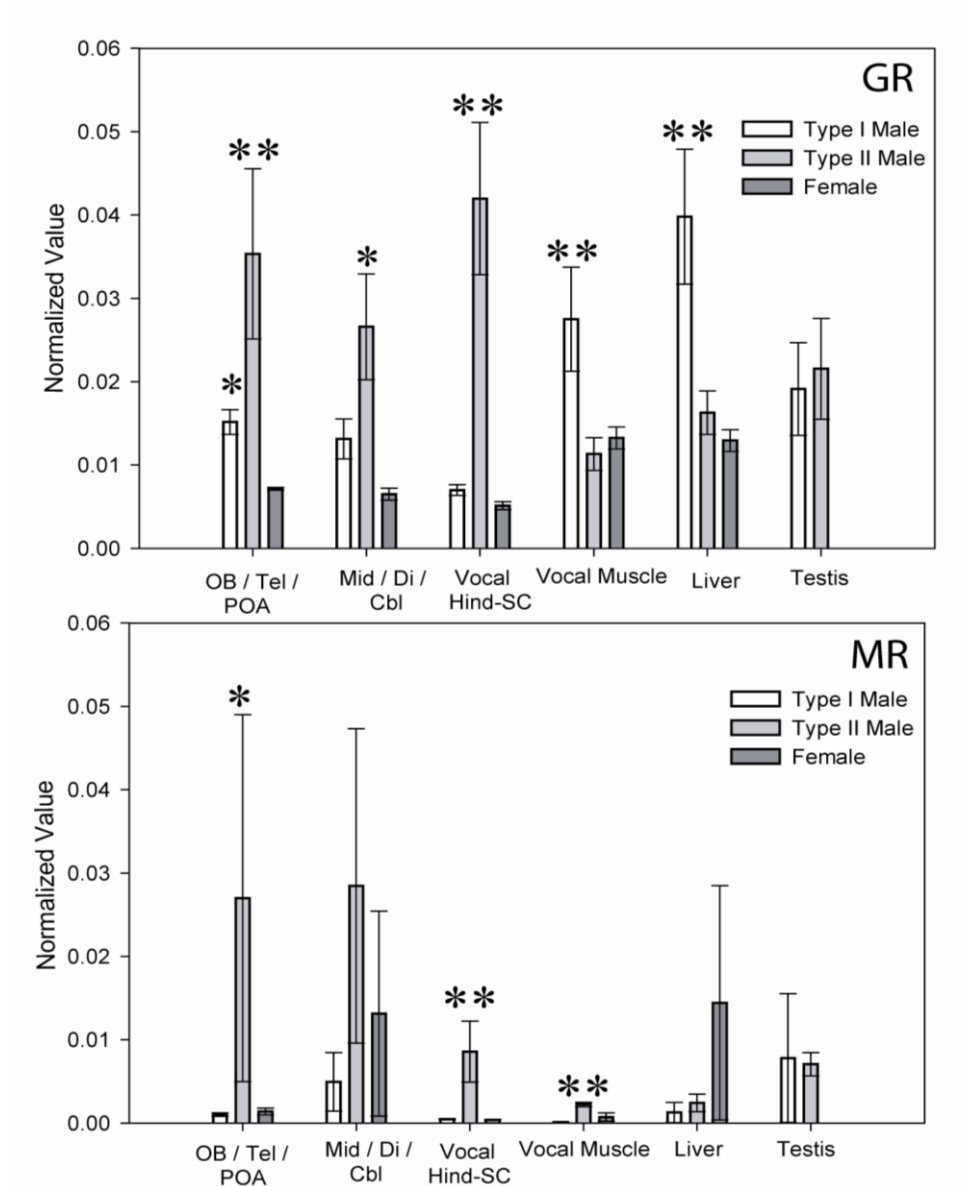


Figure 2.3: Plots showing quantitative real time PCR (qPCR) values for glucocorticoid receptor (GR, top) and mineralocorticoid receptor (MR, bottom) mRNA for each tissue sampled in the midshipman reproductive morphs (legend, top right). Values were normalized using the beta actin gene copy number from the same tissue (see Materials and Methods). Standard errors are shown and asterisks indicate significantly greater levels over one (single asterisk) or both (double asterisks) other morphs (see Supplemental Tables 3 and 4 for numerical data). Three CNS regions were sampled: forebrain including olfactory bulb (OB), telencephalon (Tel) and preoptic area (POA); a large middle region including midbrain tectum and tegmentum (Mid), diencephalon (Di) and cerebellum (Cbl); and the remaining hindbrain (hind) and rostral spinal cord (SC) that houses the vocal pattern generator circuit (Bass et al., 1994).

Table 2.3: Glucocorticoid receptor (GR) mRNA levels for tissues sampled in the three midshipman reproductive morphs. Mean normalized values (see Fig. 2.3 legend) and standard errors for mRNA levels are listed according to midshipman reproductive morph (column 1) and each tissue sampled (column 2, see Fig. 2.3 for abbreviations). ANOVA values (p value, degrees of freedom-dF, and Fratio) are also listed for each analysis performed for each tissue. Asterisks indicate significantly greater values over one or both other morphs for each tissue (see footnotes).

Morph	Breeding Season Tissue	Norm. mean	Standard error	ANOVA values
Male Type I Male Type II Female	OB/Tel/POA	0.0152* ¹ 0.0353* ¹ 0.0071	0.0015 0.0102 0.0002	p=0.0001 dF=2, 11 Fratio=22.60
Male Type I Male Type II Female	Mid/Di/Cbl	0.0131 0.0266* ² 0.0065	0.0024 0.0063 0.0007	p=0.0020 dF=2, 11 Fratio=11.55
Male Type I Male Type II Female	Vocal Hindbrain – SC	0.007 0.042* ³ 0.0051	0.0006 0.0091 0.0005	p<0.0001 dF=2, 11 Fratio=67.79
Male Type I Male Type II Female	Vocal Muscle	0.0275* ⁴ 0.0113 0.0133	0.0062 0.002 0.0013	p=0.0137 dF=2, 11 Fratio=6.49
Male Type I Male Type II Female	Liver	0.0398* ⁴ 0.0163 0.0129	0.0081 0.0026 0.0013	p=0.0011 dF=2, 10 Fratio=14.62
Male Type I Male Type II	Testis	0.0191 0.0215	0.0056 0.006	p=0.6453 dF=1, 8 Fratio=0.2287

¹II>I>F, ²II>F, ³II>I and F, ⁴I>II and F

Table 2.4: Mineralocorticoid receptor (MR) mRNA levels for tissues sampled in the three midshipman reproductive morphs. Mean normalized values (see Fig. 2.3 legend) and standard errors are listed for mRNA levels according to midshipman reproductive morph (column 1) and tissue sampled (column 2, see Fig. 2.3 for abbreviations). ANOVA values (p value, degrees of freedom-dF, and Fratio) are also listed for each analysis performed for each tissue. Asterisks indicate significantly greater values over one or both other morphs for each tissue (see footnotes).

Morph	Breeding Season Tissue	Norm. mean	Standard error	ANOVA values
Male Type I	OB/Tel/POA	0.001	0.0002	p=0.0331
Male Type II		0.027* ¹	0.022	dF=2, 10
Female		0.0014	0.0004	Fratio=4.89
Male Type I	Mid/Di/Cbl	0.0049	0.0035	p=0.4511
Male Type II		0.0284	0.0189	dF=2, 11
Female		0.0131	0.0123	Fratio=0.8565
Male Type I	Vocal Hindbrain – SC	0.0004	0.0001	p=0.0021
Male Type II		0.0086* ²	0.0037	dF=2, 10
Female		0.0004	0.0001	Fratio=12.12
Male Type I	Vocal Muscle	0.0001	0.00002	p=0.0023
Male Type II		0.0022* ²	0.0003	dF=2, 11
Female		0.0007	0.0005	Fratio=11.10
Male Type I	Liver	0.0013	0.0012	p=0.1742
Male Type II		0.0024	0.001	dF=2, 12
Female		0.0144	0.014	Fratio=2.03
Male Type I	Testis	0.0078	0.0077	p=0.0623
Male Type II		0.0071	0.0014	dF=1, 7
			Fratio=4.91	

¹II>I, ²II>F and I

In general, the expression levels of GR were significantly greater than those for MR within any one tissue for each morph ($p \leq 0.027$). GR and MR expression levels were similar, however, for the middle CNS region in type II males and females, and the liver in females ($p \geq 0.061$).

Cortisol levels

On average, RIA showed that plasma cortisol levels were more than twice as high in type II males compared to type I males: $134.96 \text{ ng/ml} \pm 21.05 \text{ (s.e.m)}$ and $48.16 \text{ ng/ml} \pm 14.13$ for, respectively, type II and type I males ($p = 0.009$; $dF = 8, 1$; $F \text{ ratio} = 11.72$). The cortisol values likely represent basal rather than stress-induced conditions related to captivity. Previous studies of midshipman show no significant differences in cortisol levels for individuals sampled either acutely or 3-4 h post-capture as was done here (20, 26). Additionally, the cortisol levels for the blood samples collected here using cardiac puncture 3-4 h after collecting type I males from nests were similar to those determined for blood samples collected from the caudal vein of type I males at the nest site (26).

Discussion

The principle findings of this investigation in the midshipman fish that has two male reproductive morphs are that (1) the GR and MR show high sequence identity with that of other vertebrate GRs and MRs and (2) there are robust morph-specific patterns in the abundance of GR and more modest differences in MR. In general, the CNS of type II males has higher levels of both GR and MR than the CNS of type I males and females. Type I males, however, show a striking increase in GR in the liver and vocal muscle as compared to type II males and females.

Vertebrate corticosteroid receptors

The consequences of corticosteroid activity are as diverse as they are necessary. Corticosteroids and their receptors have co-evolved to influence a multitude of physiological and behavioral parameters via discrete modifications in steroid signaling pathways. For example, successive mutations in key residues, such as those between GR and MR, have lead to the capacity for novel function in the face of substrate availability, termed “molecular exploitation” (2). Like tetrapods (27), two separate genes for GR and MR have now been identified in cartilaginous fish (28) and several teleost species (29, 30) that now includes midshipman fish (this report). We report one GR for midshipman as reported in several other teleosts including zebrafish (30, 31). However, as discussed earlier, we cannot yet rule out the possibility that midshipman may yet have a duplicated GR gene as in some teleosts (31-33).

Both GR and MR bind the same genomic response elements, two palindromic half-sites, but regulate separate cell processes through the interaction of their distinctive A/B domains with unique sets of transcription factors (34-36). Although the midshipman MR sequence reported here is not as complete as the GR sequence, there are two interesting differences in their amino acid sequences. S810 in human MR is important in binding aldosterone (37) and it is conserved in midshipman, but the residue is M in the midshipman GR. Similarly, Q642 in human GR is important in binding dexamethasone (38) and it is conserved in the midshipman GR, but it is an L in MR. These differences may be responsible for the ligand selectivity of the midshipman GR and MR, which is currently under investigation (A. Arterbery, A. Bass, D. Deitcher, E. Fogerty and L. Kraus, in preparation).

As in humans, cortisol is considered the principal circulating glucocorticoid in teleosts (3), including midshipman (20, 26; this report). While 11-deoxycorticosterone has been implicated in corticosteroid receptor ligand-induced activity in some teleosts

(39), it has not yet been assayed in midshipman fish. Cortisol binds to induce the transcriptional activity of both GR and MR, however, MR has a 10-fold higher sensitivity and is also activated by aldosterone (40). Aldosterone levels are typically either much lower than those for cortisol, as in some tetrapods including humans (41), or close to non-detectable as in most teleosts so far studied (22) that now includes midshipman (see Materials and Methods). Hence, MR is thought to be occupied by cortisol under basal conditions in both fish and tetrapods (40).

Divergent patterns of GR and MR expression

Collectively, qPCR and anatomical studies show that GRs and MRs are expressed throughout the body in both cartilaginous and teleost fishes (42-44). In general, GR levels were higher than MR levels in both the CNS and all of the peripheral tissues assayed in midshipman fish. Comparable studies in skates (*Leucoraja erinacea*) show that MR and GR levels are similar in the brain, while contrasting patterns are seen in peripheral tissues (28). In cichlid fish (*Astatotilapia burtoni*), MR levels are higher in the brain, while GR is higher in peripheral tissues (6).

The patterns of GR and MR expression observed in midshipman fish may reflect any number of physiological/ behavioral states (45, 46), including a wide range of factors linked to the midshipman's seasonal and daily migrations inside and outside of the intertidal zone (9). However, as noted in the Introduction, the current study investigated patterns of GR and MR expression in the context of the midshipman's two male morphs that show an extreme divergence in a wide range of traits coupled to morph-specific vocal and spawning tactics during the breeding season. Hence, we mainly focus the remainder of this section of the discussion on the potential causal relationships between patterns of receptor expression and well-established morph-

specific, reproductive-related traits in midshipman fish, fully recognizing that alternative explanations may exist.

Peripheral tissues: Vocal muscle, Liver, and Testis

The vocal repertoire of type I males is far more dynamic than that of type II males and females (see Introduction). In particular, the type I male's production of long duration advertisement hums throughout the night is likely to require intense metabolic support. Elevated GR levels in both the vocal muscle and the liver of type I males could contribute to the physiological support for prolonged vocal muscle activation. Increased GR in peripheral tissues, especially muscle, liver, and adipose tissue (47) has been associated with producing new substrate for gluconeogenesis, thereby providing glucose as an energy source (3, 48). The far more modest, but significantly elevated levels of MR in the type II muscle may yet support vocal function in type II males that has been little studied.

The vocal muscle of type I male midshipman fish exhibits a distinct suite of morph-specific traits that now include the highest GR levels, all of which may support the physiological demands of courtship calling during the breeding season (7, 14, 49). The vocal muscles of midshipman fish and other members of their family (Batrachoididae) are among the fastest contracting vertebrate muscles (12, 50). As such, the vocal muscles provide exquisite models for elucidating the potential role of corticosteroids and their cognate receptors in supporting the molecular and mechanical requirements of high speed contraction (12) that, at least in the case of midshipman, can continue non-stop for an hour or more.

Midshipman male morphs also differ in their profile of circulating steroids (20, 21, 26). The lack of any significant divergence in testis GR and MR expression between the two male morphs suggests that corticosteroids do not play a critical role

in any functions of the testis related to morph-specific patterns of steroid biosynthesis (e.g., size; see 9).

Central Nervous System (CNS)

The GR/MR expression patterns of peripheral tissues can be more directly linked to functional outcomes because of each tissue's more singular function, such as sound production by the vocal muscle (see above). The same logic can be applied to the midshipman's hindbrain-spinal cord region that is dominated by a neural circuit dedicated to vocal patterning (51, 52). Neurophysiological studies of the vocal hindbrain-spinal cord have led to several cogent hypotheses regarding the functional significance of divergent patterns of mRNA and protein abundance (14). Cortisol has morph-specific effects on vocal activity that is dependent, in part, on its direct actions on the vocal hindbrain-spinal cord (19, 53). The robust male dimorphisms in GR and MR expression in the vocal hindbrain-spinal cord parallel the morph-specific neurophysiological effects of cortisol (Fig. 2.3). Consistent with the qPCR results, we predict that anatomical studies will show a higher density of GRs and MRs in the vocal hindbrain-spinal nuclei of type II males compared to type I males, as shown for aromatase mRNA and protein (24, 54).

High and low GR mRNA levels in the mammalian CNS have been associated with, respectively, an increased and decreased display of anxiety-like behaviors (55). Similar receptor-behavioral comparisons can be made for midshipman fish. Type II males are chronically exposed to intense aggression from nest-holding type I males (11). While type I males will consistently attack type II males, the reverse is not the case; type II males successfully escape their attacks and soon return to a nest to attempt further sneak-spawnings. Compared to the territorial type I males, the anxiety-like escape phenotype of type II males is paralleled by higher CNS levels of GR/MR

and increased plasma cortisol levels. This phenotype may extend to other species with alternative male tactics. The divergent cortisol levels between midshipman male morphs resemble those of the type II-like and type I-like males in sunfish (*Lepomis macrochirus*) that have alternative reproductive tactics much like those of midshipman (56).

For type II male midshipman that exhibit the highest CNS levels of corticosteroid receptor expression among the reproductive morphs, elevated receptor expression may reflect a constitutively higher basal cortisol level that provides long-term support for their reproductive behavior and physiology. The latter relationship may reflect, more generally, a positive feedback mechanism between GR/MR and cortisol, as reported for the peripheral tissues of some teleosts (57, 58). Consistent with this interpretation, type I males showed comparatively reduced levels of both GR/MR and cortisol.

Conclusions

Vertebrates exhibit a wide range of corticosteroid-related behavioral phenotypes (45, 46). The results presented here suggest a strong relationship between peripheral and central GR/MR regulation and a suite of neural, endocrinological and behavioral traits that diverges widely between two male reproductive morphs. We propose that the co-elevation of cortisol and receptor mRNA in type II male midshipman reflects an adaptive response that supports a stable reproductive behavior, in this case sneak/satellite spawning. The opposite relationship between corticosteroid levels and receptors has been shown in mammals (including humans), where chronically elevated glucocorticoid levels are often correlated with reduced GR expression in the brain and depression (59). A more complete understanding of the molecular mechanisms leading to alternative male reproductive phenotypes like those

shown by midshipman fish can begin to contribute to a more complete understanding of the benefits and costs of therapies aimed at the maintenance of GR function and expression in the face of elevated systemic cortisol levels (59). More generally, vertebrates with alternative behavioral phenotypes (60) can now provide naturally-occurring models for identifying molecular mechanisms that promote the successful transitioning from disease to adaptive behavioral and physiological states.

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CHAPTER 3

ANDROGEN AND GLUCOCORTICOID SIGNALING MECHANISMS IN A VOCAL FISH WITH ALTERNATIVE MALE REPRODUCTIVE TACTICS

Abstract

Two enzymes that aid in both androgen and glucocorticoid production among vertebrates are 11beta (β)-hydroxysteroid dehydrogenase (11 β HSD) and 11 β -hydroxylase (11 β H). Both enzymes participate in the synthesis of two major circulating steroids in teleost fish, the glucocorticoid cortisol and the non-aromatizable androgen 11-ketotestosterone. Here, we tested the hypothesis that the abundance of messenger RNA (mRNA) for the 11 β HSD and 11 β H genes would diverge in the central nervous system (CNS) and peripheral tissues between male reproductive morphs in the vocalizing midshipman fish (*Porichthys notatus*), a teleost with alternative mating tactics. After cloning and sequencing the 11 β HSD and 11 β H genes in midshipman, quantitative real-time PCR determined their mRNA expression profiles in territorial (type I) male midshipman that acoustically court females and sneaker (type II) males that do not court females but steal fertilizations from type I males. Levels of mRNA expression were also determined for the previously cloned midshipman androgen receptor gene, a likely downstream effector of seasonal changes in androgen levels in this species. In general, type II males showed the highest expression levels for all three genes throughout the CNS. Type I males, however, had higher mRNA levels for all genes in the vocal (sonic) muscle, for 11 β HSD and 11 β H in the testis, and for 11 β HSD in the liver. Together, the expression patterns show that

mRNA abundance for the enzymes and receptors mediating androgen and glucocorticoid signaling pathways in type II males are distinct from those of type I males (and females), reflecting their equally distinct vocal, spawning and endocrine phenotypes.

Introduction

Steroid biosynthesis is an ancient and crucial mechanism among vertebrates for regulating physiological processes across multiple organ systems (1, 2). One enzyme that is responsible for oxidation and reduction at the carbon-11 position of the cholesterol ring is 11beta (β)-hydroxysteroid dehydrogenase (11 β HSD). Among teleost fish, 11 β HSD along with 11 β -hydroxylase (11 β H) aids in the conversion of testosterone to 11-ketotestosterone (11KT), an end-product in the androgen pathway (see Fig. 3.1 and 3). Like humans, 11- β HSD in fish also inter-converts cortisol with its inactive metabolite, cortisone (see Fig. 3.1 and 3). In fish, elevated plasma levels of 11KT and cortisol are often reported during the breeding season and accompany changes in a large suite of neuroendocrinological and behavioral traits (4, 5). While some mammals apparently produce low levels of 11KT, a mechanism was only recently proposed that included 11 β HSD acting on androgens in the murine gonad (6). Thus, the mechanisms regulating both androgen and glucocorticoid metabolites in fish may be more widespread than previously thought.

Since the enzymes 11 β HSD and 11 β H regulate both androgen and glucocorticoid synthesis, they may also contribute to the regulation of reproductive-related neural and behavioral events. Here, we investigated mRNA expression levels for the 11 β HSD and 11 β H genes (respectively, 11 β HSD1 and CYP11B) in the midshipman fish (*Porichthys notatus*), a sound-producing/ vocal fish with two male





Glucocorticoid Synthesis	Enzyme	Androgen Synthesis
11 - Deoxycortisol  Cortisol  Cortisone	11βH 11βHSD	Testosterone  11 β - (OH) Testosterone  11-Ketotestosterone

Figure 3.1: Overview of synthetic pathways for glucocorticoids and androgens in fish. See text and Bury and Sturm (2007) for details.

morphs that diverge in reproductive tactics and where androgens (including 11KT) and cortisol, the main glucocorticoid (7), have male morph- and sex-specific effects on reproductive behavior and vocal traits (8, 9). Androgen receptor (AR) mRNA abundance was also studied given its prominent expression in the vocal muscle and central vocal regions in midshipman (10), and its being a likely effector of androgen action (11, 12). A previous study cloned, sequenced and characterized mRNA levels of the genes encoding midshipman corticosteroid receptors (13), likely targets of cortisol.

The vocal muscles of type I males have several traits that are greater in magnitude than those of type II males and females (which are similar to each other), ranging from muscle mass to the width of a myofibril's Z-lines and the abundance of aerobic enzymes (14, 15). Similar dimorphisms characterize the sizes of neurons within a vocal pattern generator that spans the caudal hindbrain and rostral spinal cord (16). In sum, the structural and biochemical attributes of the type I male's vocal system reflect its adaptation to the performance of a metabolically demanding behavior, long duration advertisement calling.

Like other species with alternative male reproductive tactics (5, 17), midshipman exhibit male morph and sex-specific profiles in plasma levels of steroid hormones (5, 7, 17, 18). During the pre-nesting and nesting periods of the breeding season (see 7) for definitions), the main circulating androgen in type I males is 11KT, with plasma levels almost 10 fold that of testosterone; 17 β -estradiol is often undetectable (7, 18, 19). 11-Ketotestosterone is essentially undetectable in type II males and females, while testosterone is the predominant androgen in both of these morphs. Females alone show 17 β -estradiol levels that may be 50% more than testosterone (above references).

The androgen and glucocorticoid-dependent sensitivity of the vocal system is

well-established (9). Both testosterone and 11KT influence vocal muscle and motoneuron size (8, 20, 21) and, along with cortisol, have sex- and morph-specific effects on the firing pattern of the hindbrain-spinal vocal pattern generator (12, 22). These findings, along with the dramatic sexual polymorphisms in vocal and neuronal traits (see above references), led to the current study testing the hypothesis that mRNA expression patterns of components in the androgen and glucocorticoid signaling pathways (AR, 11 β HSD, 11 β H) would diverge in parallel with vocal, reproductive and neuroendocrine phenotypes in midshipman fish.

Materials and Methods

Animals

Midshipman fish were hand-collected from field sites in northern California during the breeding season (May-August), shipped to Cornell University within 72 h, and maintained in seawater tanks until sacrificed for tissue within 24 – 48 h of receipt. This study included type I males (20.6 – 25.1 g, 12 - 13.5 cm in standard length), type II males (3.94 - 5.88 g, 8 - 8.5 cm) and females (11.1 - 17.5 g, 10 - 11.5 cm). The gonad and vocal muscle of the animals were used to verify sex and male morph status (23). Tissue sampling was carried out following deep anesthetization (0.025% benzocaine; Sigma, St. Louis, MO). All experimental protocols were approved by the Cornell University Institutional Animal Care and Use Committee.

Cloning, Sequencing, and Alignment

RNA was isolated and pooled from type I male whole CNS (n = 3 individuals) from the breeding season using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) and random

hexamers following the manufacturer's protocols. Central nervous system (CNS) samples included both the brain and the rostral spinal cord that includes the caudal part of the vocal pattern generator (9, 24). PCR on cDNA from all tissues were conducted using the following degenerate primers: 11 β HSD forward primer (SNYRGCME): TCN-AAY-TAY-AGR-GGN-TGY-ATG-GAR, 24 nucleotides, T_m = 55.8°C; 11 β HSD reverse primer (ETKDLFQ): GAR-ACN-AAR-GAY-CTN-TTY-CAR, 21 nucleotides, T_m = 50.5°C; 11 β H forward primer (GVFLKNG): GGN-GTN-TTY-CTN-AAR-AAY-GGN, 21 nucleotides, T_m = 53.5°C; 11 β H reverse primer (ANITEL): GCN-AAY-ATH-ACN-GAR-CTN-ATG, 21 nucleotides, T_m = 51°C. These primers were designed to amplify a highly conserved region based on an amino acid alignment of vertebrate steroidogenic enzymes. Amplification was performed with *Taq* polymerase (Epicentre, Madison, WI) using the following cycles: Stage 1 – 94°C for 2 min (1 cycle), Stage 2 – 94°C for 1 min, 52°C for 1 min, 72°C for 3 min (35 cycles), and Stage 3 – 72°C extension for 10 min (1 cycle). Products were run on a 1% agarose gel and verified to be the approximate expected size: a 380 bp product for 11 β HSD and a 595 bp product for 11 β H. The primers contained restriction sites on the 5' end to increase the efficiency of ligation into the Bluescript KS (+) plasmid, and the ligations were transformed into DH5 α (Invitrogen, Carlsbad, CA) competent cells and plated. Twenty individual clones were grown in liquid culture, mini-prepped (Qiagen, Germantown, MD), and sequenced at the Cornell University Life Sciences Core Laboratory Center using T3 and T7 primers.

The resulting sequences were subject to a BLAST analysis (NCBI) to verify identity and an alignment for vertebrate comparison (CLustalW). Sequences for both alignments were obtained from the following species: **11 β HSD** *Homo sapiens* 11 β HSD 1 (Genbank accession number NM_005525.2), *Mus musculus* 11 β HSD 1 (Genbank accession number NM_001044751.1), *Homo sapiens* 11 β HSD 2 (Genbank

accession number NM_000196), *Mus musculus* 11 β HSD 2 (Genbank accession number NM_008289.2), *Taeniopygia guttata* 11 β HSD 2 (Ensembl identifier ENSTGUG000000005862), *Anolis carolinensis* (Ensembl identifier ENSACAG000000003658), *Xenopus tropicalis* 11 β HSD 2 (Ensembl identifier ENSXETG000000004704), *Danio rerio* (Genbank accession number NM_212720), *Oreochromis niloticus* (Genbank accession number AY190043.2), *Anguilla japonica* (Genbank accession number AB252646), *Oncorhynchus mykiss* (Genbank accession number AB104415); **11 β H** *Homo sapiens* 11 β H2 (Genbank accession number NM_000498), *Mus musculus* 11 β H2 (Genbank accession number NM_009991.3), *Homo sapiens* 11 β H1 (Genbank accession number NM_000497), *Mus musculus* 11 β H1 (Genbank accession number NM_001033229.2), *Anolis carlinensis* (Ensembl identifier ENSACAG000000004353), *Xenopus tropicalis* (Ensembl identifier ENSXETG000000001446), *Acanthopagrus schlegelii* (Genbank accession number EF423618), *Dicentrarchus labrax* (Genbank accession number AF449173). There was neither an annotated nor a predicted protein sequence for zebra finch 11 β H in Genbank or Ensembl.

As noted earlier, a prior study cloned, sequenced, and compared the androgen receptor in midshipman (10; P. Forlano, M. Marchaterre, D. Deithcer, A. Bass, to be submitted).

Absolute-Quantitative Real Time PCR (qPCR)

RNA from several CNS regions and peripheral tissues (see below) was isolated from individual type I males, type II males and females (n = 4 – 5 individuals/ morph) using Trizol (Invitrogen, Carlsbad, CA), treated twice with DNaseI (Invitrogen, Carlsbad, CA) to remove genomic DNA contamination, and reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the

manufacturer's protocols. As in previous biochemical and mRNA expression studies (25), the CNS was divided into three major regions: a forebrain region that included the olfactory bulb, telencephalon and preoptic area; a middle region including midbrain tectum and tegmentum, diencephalon, and cerebellum; a caudal region including the remaining hindbrain and rostral spinal cord that includes the vocal pattern generator region (24). The forebrain and hindbrain-spinal regions assayed here were focused upon because of their known role in vocal motor patterning (9, 26) and their abundance of estrogen and androgen receptor mRNA, aromatase mRNA and neuropeptide-containing neurons (8, 9, 26-28). Samples were also taken from liver, testis and vocal muscle for reasons stated above. Peripheral tissues sampled included the testis because of its role in steroid biosynthesis and the vocal muscle because of its essential role in sound production. Unlike the other tissues sampled that have known dimorphisms in midshipman fish, we also examined gene expression in the liver because of its essential role in metabolic function and its wide use in other studies of gene expression in teleosts (including midshipman, (29, 30) and vertebrates in general. The ovary was not assayed because of the main focus here on divergence in mRNA expression between the male morphs.

Absolute qPCR was conducted on cDNA from all tissues using gene specific primer pairs that were chosen based on the following criteria: 1) amplify a product about 150 nucleotides in length, 2) isolate a single product with no dimer pairs, and 3) have a standard efficiency (R^2) of no less than 98%. The following primers were designed from the nucleotide sequence of the midshipman genes: For the **11 β HSD**, forward primer (5' CCGAGCCTCCGTCATTCTGC, 20 nucleotides, 60.6°C) and reverse primer (5' CGTGACTGAGACCAAGGACCTCTTT, 24 nucleotides, 60.2°C) were chosen as the primer pair ($R^2=0.982$); For the **11 β H gene**, forward primer (5' CGTGCCCCCTTGTGGACA, 18 nucleotides, $T_m = 60.8^\circ\text{C}$) and reverse primer

(5' AAGGACACCAGTACACCGGAGTT C, 24 nucleotides, $T_m = 60.4^\circ\text{C}$) were chosen as the primer pair ($R^2=0.987$); For the **AR** gene (Genbank accession No. DQ109666), forward primer (5' GCTGGAATGACCCTTG GAGCA, 21 nucleotides, $T_m = 59.9^\circ\text{C}$) and reverse primer (5' TCAACTCCCACGTGGTCTTCCTC, 23 nucleotides, $T_m = 60.6^\circ\text{C}$) were chosen as the primer pair ($R^2=0.98$). **Beta actin** primers were designed from *Acanthopagrus schlegelii* (Genbank Accession No. AY491380) and forward primer (5' GGT GATGGTGTGACCCACACAGTG, 18 nucleotides, $T_m = 60.7^\circ\text{C}$) and reverse primer (CTCCGATCCAGACAGAGTATTTAC, 20 nucleotides, $T_m = 60.1^\circ\text{C}$) were chosen for the primer pair ($R^2=0.988$).

All real time reactions were run in triplicate for the above tissue samples and no template controls, and contained the following: 10 μl of 2X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 2 μl of forward and reverse primer at a concentration of 100 nM, 4 μl of H_2O , and 2 μl of the appropriate cDNA. Reactions were run on an applied Biosystems 7900 HT Sequence Detection System at the Cornell University Life Sciences Core Laboratory Center under the default manufacturer's conditions (SDS 2.1 software, Applied Biosystems, Foster City, CA) using 60°C as an annealing temperature.

Gene copy number was determined using standard curve analysis for all gene primer sets, including beta actin. The standards covered a linear range of 5×10^6 to 100 copy/ μl . Briefly, the raw C_t values were converted to copy number with the standard curve produced using the SDS 2.1. Each target gene copy number was normalized using the beta actin copy number from the same tissue sample. The normalized values were determined for each individual.

Statistics

Normalized mRNA values were compared between morphs for each tissue sampled. Statistics were performed on all data sets using ANOVA analysis and post-hoc Tukey-HSD tests. Statistics were performed using JMP 7.0 software. In addition, analysis of variance was performed for each comparison and Welch-ANOVA tests conducted on those with unequal variance to verify any significant result.

Results

Cloning, Sequencing, and Alignment

Realizing that androgens and glucocorticoids play a prominent role in the differential seasonal behavior of midshipman (see Introduction), we investigated the expression of synthetic enzymes involved in the synthesis of both classes of steroids. We first identified conserved blocks of amino acids in vertebrates for the synthetic enzymes 11 β HSD and 11 β H and then designed degenerate primers to those regions to amplify PCR products from cDNA of type I male CNS. The resulting PCR products of 380 bp for the 11 β HSD gene (HSD11B) and 595 bp for the 11 β H gene (CYP11B) were subcloned and sequenced. Degenerate primers revealed a single amplification product for both the 11 β HSD and 11 β H genes, and gene-specific primers also revealed a single product as expected. The nucleotide sequence translated protein sequence can be viewed on Genbank on the NCBI webserver (Genbank Accession No. EU530638 for HSD11B and EU869313 for CYP11B).

Alignments of the midshipman enzymes with other vertebrate enzymes revealed high sequence identity (alignment in Fig. 3.2 and sequence identity in Table 3.1 for 11 β HSD; alignment in Fig. 3.3, and sequence identity in Table 3.2 for 11 β H). As depicted for 11 β HSD (Fig. 3.2), this sequence includes properties that allow access

Figure 3.2: An alignment of deduced amino acid sequence of midshipman (*P. notatus*) and other vertebrate 11 β HSD enzymes reveals high sequence identity between mammals and teleost fishes. Black and gray shading indicate identical and similar amino acids, respectively. Both the forward and reverse primers used in qPCR for the midshipman 11 β HSD are underlined.

H.sapiens HSD1 1 -----MAFMKKYLLPLLGTFMAYYYSSAN
M.musculus HSD1 1 -----MAVMKNYLLPLLVLFAYYYSTN
H.sapiens HSD2 1 -----MERWP--WPSGCAWLLVAARAILQLLRSDRLGRPLLAALALLAANWLCQRLPPPAATLAVIAAGWIALSR
M.musculus HSD2 1 -----MERWP--WPSGCAWLLVAARAILQLLRSDRLGRPLLAALALLAANWLCQRLPPPAATLAVIAAGWIALSR
T.guttata HSD2 1 -----LGRGLVLPALLAALQSLCRAWLPLEPLGLAAAGAGLLWR
A.carolinensis HSD2 1 -----
X.tropicalis HSD2 1 -----MELAACPSIMAYGVVLSFLLFLFRFSNDVLSPALLLYVVVLVLEWLCCHLYPIALGLIFSSACWYVLGI
D.rerio 1 -----MSIFVCGAKKKFLAFNGAMPSSVVVWLGAATLLVERLCALCPAVALRLVLCVCCWYFTW
O.niloticus 1 -MEDYTLP-----FWIYLDIVTVFICGAMKKILASHNATSTVVAWLGAATLVVERLWAFCPAALLLVFGITFCYYAT
A.japonica 1 MIPNAIEDCALSEFWIYVVMVSVFVGAMKKILASHSAAPTVAWLGAATLLVERLCSLWEPALLALAVCAVCCCLSVR
O.mykiss 1 -MEDYLD-----WIYVMSAFVCGAMKKVLASHSAAPTVAWLGAATLLVERLWFFCPAALLVAAALCLACCCYSVR
P.notatus 1 -----

H.sapiens HSD1 25 ---EEFRPEMTCGKKVITGASKGIGREMAVHLAKMCAHVVTARSKET-----LQVVSHCLPLGAASAHYIAGTMEDM
M.musculus HSD1 25 ---EEFRPEMTCGKKVITGASKGIGREMAVHLAKMCAHVVTARSEE-----LQVVSHCLPLGAASAHYIAGTMEDM
H.sapiens HSD2 72 --LARPQLPVATHAVITGCDSGFGKETAKLDSMGFTVLATVLEINSPGATELRCCSRLRLQMDITKPGDISRVL
M.musculus HSD2 72 --LARPPRLPVATHAVITGCDIGFGKETAKLDSMGFTVLATVLDLNSPGATELRDCCSRLRLQMDITKAEDISRVL
T.guttata HSD2 43 --AARGLLPVAGHAVITGCDSGFGQATAHLDIMGERVFAVLDLQSPGACELRQSCSRLRLQMDITKTEDICRVL
A.carolinensis HSD2 1 -----GCDSSGFGKAAAHLDSELRVFATVLDLQSPGAEELRSCSRLRLQMDITQEEDIQRAV
X.tropicalis HSD2 76 --VSKRTLPEVGRVHVITGCDSSGFGNVAAKLDSMGFKVIATVLEINLESPGARKLRKICSDNLTITQMDITKQEDIKRAQ
D.rerio 61 --ATKPKSLPVEDKAVITGCDSSGFGNATAKLDSMGFEVFATVLEINLESPGAKHLRVCSSRLRLQMDITQPPQOQCAL
O.niloticus 75 KTSQPRAMLPAHGKAVITGCDSSGFGNATAHLDSGFEVFATVLDLNGDGAKELOKTCSPHLLRLQMDITQPPQOQCAL
A.japonica 81 --GGPARTLPADGKAVITGCDSSGFGKATAHLDAGFEVFAVLDLDGPGATELRACSSRLRLQMDITQPPQOQCAL
O.mykiss 73 --TAPATLPVCGKAVITGCDIGFGKAAARLDAQGFEVFATVLDLCEGAKELOKTCSPHLLRLQMDITQPPQOQCAL
P.notatus 1 -----

GxxxGxG

H.sapiens HSD1 97 TFAEQVYQAGKLMGGLDMLILNHITNTSLNFHDDIHVVRKSMEVNFSLYVLTVAALPMLQSNISIVVSSLAGKVA
M.musculus HSD1 97 TFAEQVYKAGKLMGGLDMLILNHITQTSLSLFHDDIHSVVRVMEVNFSLSYVMTALPPLQSNISIAVSSLAGKVT
H.sapiens HSD2 150 EFTKAHTTSTGLWGLVNNAGHNEVADAELESPAT-----YRSCMEVNFFGALELTGKLLPLLRSSGRIVTVGSPAGDMF
M.musculus HSD2 150 EITKAHTTST-----VADAELESPAT-----YRSCMEVNFFGALELTGKLLPLLRSSGRIVTVGSPAGDMF
T.guttata HSD2 121 QHICAHNTNSTGLWGLVNNAGFNDDTDAEELSPLGK-----YRSCMEVNFFGSLLETKGLLPLLRSSGRIVTVGSPAGDMF
A.carolinensis HSD2 62 GFVHAOTGTGLWGLVNNAGWNTTDAELELEPE-----YRYCMEVNFFGTALTGKLLPLLRSSGRIVTVGSPAGDMF
X.tropicalis HSD2 154 QTKRLHTAGRLWLLNNAGYCAHTDAEELSLSST-----YRSCMEVNFFGTLELTGKLLPLLRSSGRIVTVGSPAGHFS
D.rerio 139 LDTKAKLIRLWGLVNNAGWCVNITDAELSLSMS-----YRSCMEVNFFGTITTTTLETLPLLRQSGRIVTVSSPAGHFP
O.niloticus 155 LDTKAKLCLRLWGLVNNAGVCVNFEDAELSLSMS-----YRSCMEVNFFGTLSITKLETLPLLRQSGRIVTVSSPAGQCF
A.japonica 159 LATKAQCLRLWGLVNNAGVCVNITDAELSLSMS-----YRSCMEVNFFGTINVTKTLETLPLLRQAGRIVTVSSPAGQCF
O.mykiss 151 LDTKAKLCLRLWGLVNNAGVCVNFDAELSLSMS-----YRSCMEVNFFGTINVTKTLETLPLLRQSGRIVTVSSPAGQCF
P.notatus 1 -----SN-----YRSCMEVNFFGTPSMTOTLETLPLLRQSGRIVTVSSPAGQCF

5' cloning primer

H.sapiens HSD1 177 YEMVAAYSASKFALDGFSSIRKEYSVSRVNVSTLCVLGLIDTEAMKAVSGIVHMQAPKEECALEIKGGAHQOEVEV
M.musculus HSD1 177 QEMVAAYSASKFALDGFSSIRKEYSVSRVNVSTLCVLGLIDTEAMKEISGIINAQASPKKEECALEIKGTARKSEV
H.sapiens HSD2 226 YPCLAAYGTSKAAALMDTFSCGLPWGVKVSIIQFGCKTESVRNVGVQWEKRLQLLANLPQELLQAYGKYEHVHIG
M.musculus HSD2 212 YPCLAAYGTSKAAALMDTFGCGELPWGVKVSIIKFGCKTKTDAVINNVNLWEKRLQLLANLPRELLQAYGEYEHVHIG
T.guttata HSD2 197 FPCLAAYGASKAALSLMDTFRSELCPWGVKVSIIIPGYKYKTCTCDPAFNNLQOQLLASLPQELLQAYGEYEHVHINQ
A.carolinensis HSD2 138 YPCLASYGASKAALSLMDTFRCGLAPWGVKVSIIIPGYKYKT-----
X.tropicalis HSD2 230 FFLAAAGSSKAAALNMDTFRHELPWGVKVSIIIPASAYKTGAHDNHIHWNQKKLLANLPIELLOBYGEYETETON
D.rerio 215 FPCLASYGASKAALNMDTFRHELPWGVKVSIIIPASAYKTGSSNAEYWEKQYKSLLOGLSPNLEFYGEYETETNE
O.niloticus 231 FPCLAAYGASKAALNMDTFRHELPWGVKVSIIIPSSYKTGSSNSAEYWEKQYKHLLOQLSPALLEFYGEYETETND
A.japonica 235 FPCLAAYGASKAALNMDTFRHELPWGVKVSIIIPSAKTGSSNFEYWEKQYKFNQNLSPALLEFYGEYETETNE
O.mykiss 227 FPCLAAYGASKAALNMDTFRHELPWGVKVSIIIPSSYKTGSSNQBYWENQYKSLIHLNLTGLLEFYGEYETETNE
P.notatus 44 FPCLAAYGASKAALNMDTFRHELPWGVKVSIIIPSSYKTGSSNNAYWBOQYKQLOLSHPALLEFYGEYETETND

YxxxK

5' qPCR primer

3' qPCR primer (bottom)

H.sapiens HSD1 257 YND-----SSTWTTLLIRNF-----CRKILEFLNISTSYN-----MDRFYFNK-----
M.musculus HSD1 257 YND-----KSLPTEILGNP-----CRKIMEFSLRYYN-----KDYFUSN-----
H.sapiens HSD2 306 QELHSLRLAMSDLPVDAITDALLAARERRRYFEGGLGLMYFIHHYFPEGRRRFLQAFETIS-HCLPRALPQPGQGT
M.musculus HSD2 292 QELNSLRMALPDLSPVDAITDALLAARSRRYFEGGLGLMYFIHHYFPEGRRRFLQAFETIS-HCLPRALPQPGQGT
T.guttata HSD2 277 QETQFMVAVEDLSAVVNSITBGLLAANAVRYFEGGLGLMYFIHRYLPEYFRLFLFKGFFN-PKLPRALA-----
A.carolinensis HSD2 -----
X.tropicalis HSD2 310 RELKYGDTACSLSPVNDISITDAISENKKVYYAGKNLWLYLIGIYLFHSVSDNFCCKCFFKNNVLPRLSKQKTNE-
D.rerio 295 LEQNYATANEDLSPVNDITVBALLSPQVRYAYAGGLILMYFICSYPPLSISDRFLQKLFVQKKVMPRALIKQOGLSP
O.niloticus 311 LEQSFARHTTTNLPVDAITIVQALLAPQOPRYFAGSGLSLMYFIHSYFSPYSISNFFLKKRFFKKNVLPRLAKQSAFDV
A.japonica 315 LEQGHSTAPEDLSPVNDITDALLSLREKVRYSCEGGLMYFIYNYFPLSDYFLQRLFFVKKKVIPLRLAKQSIINN
O.mykiss 307 LEQHFQSANEDLSPVNDITBALLSPRELVRYYAGGGLMNFYYSYFPLSDYFLQRLFFVQKKIPLRLAKKE
P.notatus 124 LEQ-----

3' cloning primer (top)

H.sapiens HSD1 -----
M.musculus HSD1 -----
H.sapiens HSD2 385 PPQDAAQDPNLSFGPSPAVAR-----
M.musculus HSD2 367 -----HGPAFA-----
T.guttata HSD2 -----
A.carolinensis HSD2 -----
X.tropicalis HSD2 -----
D.rerio 375 NDNNSIKENMNDSSNNSNFTKCID
O.niloticus 391 NLSLHNNNE-----EKLOQM
A.japonica 395 NNNNNNNNNNNNNNNNDINKID--
O.mykiss 382 NNNNNNNNNN-----VEKIKK--
P.notatus -----

Table 3.1: Sequence identity chart for all sequences used in the 11 β HSD alignment.

	H.Sapiens 11 β HSD 1	H.Sapiens 11 β HSD 2	M.musculus 11 β HSD 1	M.musculus 11 β HSD 2	T.guttata 11 β HSD 2	X.tropicalis 11 β HSD 2	A.carolinensis 11 β HSD	D.reio 11 β HSD	O.nitobius 11 β HSD	A.japonica 11 β HSD	O.mykiss 11 β HSD	P.nictatus 11 β HSD
H.Sapiens 11 β HSD 1	100		78		13	16	26	18	19	15	20	26
H.Sapiens 11 β HSD 2	16	100	15		64	44	70	41	40	43	41	49
M.musculus 11 β HSD 1	78	15	100		13	15	25	19	20	16	20	25
M.musculus 11 β HSD 2	17	66	15	100	60	45	62	42	43	45	42	50
T.guttata 11 β HSD 2	18	64	18	60	100	40	75	48	46	52	49	57
X.tropicalis 11 β HSD 2	16	44	15	45	40	100	57	48	45	48	46	57
A.carolinensis 11 β HSD	26	70	25	62	75	57	100	59	59	66	60	42
D.reio 11 β HSD	18	41	19	42	48	48	59	100	64	69	71	78
O.nitobius 11 β HSD	19	40	20	43	46	45	59	64	100	64	64	82
A.japonica 11 β HSD	15	43	16	45	52	48	66	69	64	100	76	73
O.mykiss 11 β HSD	20	41	20	42	49	46	60	71	64	76	100	80
P.nictatus 11 β HSD	26	49	25	50	57	57	42	78	82	73	80	100

Figure 3.3: An alignment of deduced amino acid sequence of midshipman (*P. notatus*) and other vertebrate 11 β H enzymes reveals high sequence identity between mammalian and teleost species. Black and gray shading indicate identical and similar amino acids, respectively. Both the forward and reverse primers used in qPCR for the midshipman 11 β H are underlined.

H.sapiens 11bH2 1 -----MALRAKAEVCTAAPWLSLQRAALGTRAARAPRTLPFEAMFQHPGNRW
M.musculus 11bH2 1 -----MALRVTDVWVARPWQCLHRTALGTTATLAPKTQPFEAMFQYSRNKW
H.sapiens 11bH1 1 -----MALRAKAEVCTAAPWLSLQRAALGTRAARVPRTLFPFEAMFRRPFGNRW
M.musculus 11bH1 1 -----MTMALRVTTDVMVARPWQCLHRTALGTTATLAPKTQPFEAMFQYSRNKW
A.carolinensis 1
X.tropicalis 1
A.schlegelli 1
D.labrax 1 MRSMYIRVTVCIRAQGTGRSNNLLCGAAPQALCLTAAGTVVDGKLEGRKRGESTGVRRKGVDRGRSFEELFHTGRNGW
P.notatus 1 -----

H.sapiens 11bH2 50 LRLQIWRQGYEDLHLEHQTFOELGPIRYNNGGPRMVCMLFEDVEKLCQVDSLHPERMILEPWVAYRQHRGHKCGV
M.musculus 11bH2 50 LKMIQILREQGENLHLEHQTFOELGPIRRHSVCKTQIVSNMLEDVAKLHQVDSLPMRMRHLEPWVAHRELRLGRGV
H.sapiens 11bH1 50 LRLQIWRQGYEDLHLEHQTFOELGPIRYDGGAGMVCMLFEDVEKLCQVDSLHPERMILEPWVAYRQHRGHKCGV
M.musculus 11bH1 52 LKMIQILREQGENLHLEHQTFOELGPIRRHSVCKTQIVSNMLEDVAKLHQVDSLHPERMILEPWVAYRQHRGHKCGV
A.carolinensis 1
X.tropicalis 1
A.schlegelli 1
D.labrax 81 VNIVKFFREDRFRHLHKKHVERTENALGPIREHYGTQSSVNMLESIDIGELFRAGLHFERMTLQWATHREIRQHSKV
P.notatus 1 -----GV

H.sapiens 11bH2 130 FLNNGEWFNRLRLNPNVSPKAVQFPLPMVDAVARDESQALKKVLQNRG-----SLTLVQCSIFHYTTEASNLAL
M.musculus 11bH2 130 FLNNGEWFNRLRLNPNVSPKAVQFPLPMVDAVARDELTLLKPKVLQNRG-----SLTMVQCSLNYTTEASNFAL
H.sapiens 11bH1 130 FLNNGEWFNRLRLNPNVSPKAVQFPLPMVDAVARDESQALKKVLQNRG-----SLTLVQCSIFHYTTEASNLAL
M.musculus 11bH1 132 FLNNGEWFNRLRLNPNVSPKAVQFPLPMVDAVARDEVDNLKKVLESVHG-----SFSMDQSSVFNHYTEASHFVL
A.carolinensis 2 LCRNGEKWRSRLNPNVSPKAVQFPLPMVDAVARDEVDNLKKVLESVHG-----RSLTLVQCSIFHYTTEASNLAL
X.tropicalis 1
A.schlegelli 3 FLNNGEWFNRLRLNPNVSPKAVQFPLPMVDAVARDECRMLRAKVEREGRGEGRRSLTMPSDPLFRFAEASCHVT
D.labrax 161 FLNNGEWFNRLRLNPNVSPKAVQFPLPMVDAVARDECRMLRAKVEREGRGEGRRSLTMPSDPLFRFAEASCHVT
P.notatus 3 FLNNGEWFNRLRLNPNVSPKAVQFPLPMVDAVARDECRMLRAKVEREGRGEGRRSLTMPSDPLFRFAEASCHVT

5' cloning primer
H.sapiens 11bH2 205 EGERLGLVGHFSSASINFLHAEVMEKSTVQLMFPSPSLRWISPKVWKHHFAWDCEIQYGDNCIOKIYQELAFNRPO
M.musculus 11bH2 205 EGERLGLVGHFSSASINFLHAEVMEKSTVQLMFPSPSLRWISPKVWKHHFAWDCEIQYGDNCIOKIYQELAFNRPO
H.sapiens 11bH1 205 EGERLGLVGHFSSASINFLHAEVMEKSTVQLMFPSPSLRWISPKVWKHHFAWDCEIQYGDNCIOKIYQELAFNRPO
M.musculus 11bH1 207 EGERLGLVGRDLSPLSLKFLHTHSMEKTTTOLLYLPLSLTRWTSRVRWKHNLSDWDFISEYVTKCIKNVYRELAEGRPQ
A.carolinensis 77 YGERLGLVEENPNPSSKFIWAVETMTOTLPLLLPPLPGVVRWVSPKLWQDHMDAWDAIHAHAKCIONDYQEFCLGQPR
X.tropicalis 7 YGERLGLVEENPNPSSKFIWAVETMTOTLPLLLPPLPGVVRWVSPKLWQDHMDAWDAIHAHAKCIONDYQEFCLGQPR
A.schlegelli 83 YGERLGLFSSSPSSKFIWAVETMTOTLPLLLPPLPGVVRWVSPKLWQDHMDAWDAIHAHAKCIONDYQEFCLGQPR
D.labrax 241 YGERLGLFSSSPSSKFIWAVETMTOTLPLLLPPLPGVVRWVSPKLWQDHMDAWDAIHAHAKCIONDYQEFCLGQPR
P.notatus 83 YGERLGLFSSSPSSKFIWAVETMTOTLPLLLPPLPGVVRWVSPKLWQDHMDAWDAIHAHAKCIONDYQEFCLGQPR

5' qPCR primer
H.sapiens 11bH2 285 -----HYTGIAELLKAEFLSLAIKANSMEITAGSVDTTATPLMTLFEELARNPVQQLRQESIAAAASISEHP
M.musculus 11bH2 285 -----TYSGIAELLKAEFLSLAIKANSMEITAGSVDTTATPLMTLFEELARNPVQQLRQESIAAAASISEHP
H.sapiens 11bH1 285 -----QYTSIAELLKAEFLSLAIKANSMEITAGSVDTTATPLMTLFEELARNPVQQLRQESIAAAASISEHP
M.musculus 11bH1 287 -----SHEIAELLKAEFLSLAIKANSMEITAGSVDTTATPLMTLFEELARNPVQQLRQESIAAAASISEHP
A.carolinensis 157 -----KYSIAELLKAEFLSLAIKANSMEITAGSVDTTATPLMTLFEELARNPVQQLRQESIAAAASISEHP
X.tropicalis 87 -----GYSIAELLKAEFLSLAIKANSMEITAGSVDTTATPLMTLFEELARNPVQQLRQESIAAAASISEHP
A.schlegelli 163 GAEAGAAGGRYTGVLGQLMKGGQLSLDLIKANTELMAAGGVDTTAMPLQFALFELGRNPVQQRVCHVRVSWAQAGSDP
D.labrax 321 GSKAGAPGAQYTGVLGQLMKGGQLSLDLIKANTELMAAGGVDTTAMPLQFALFELGRNPVQQRVCHVRVSWAQAGSDP
P.notatus 163 VSEHGAEGHQYTGVLGQLMKGGQLSLDLIKANTELMAAGGVDTTAMPLQFALFELGRNPVQQRVCHVRVSWAQAGSDP

3' qPCR primer 3' cloning primer
H.sapiens 11bH2 356 QKATTELPLLRRAAKETRLRYPVGLFTEFVVSDDIVLQNYHPAGTLVQVFLYSVGRNPAFFRPERYMPQRWLDFG--
M.musculus 11bH2 356 QKASDLPLLRAAKETRLRYPVGLFTEFVVSDDIVLQNYHPAGTLVQVFLYSVGRNPAFFRPERYMPQRWLDFG--
H.sapiens 11bH1 356 QKATTELPLLRRAAKETRLRYPVGLFTEFVVSDDIVLQNYHPAGTLVQVFLYSVGRNPAFFRPERYMPQRWLDFG--
M.musculus 11bH1 357 QKASDLPLLRAAKETRLRYPVGLFTEFVVSDDIVLQNYHPAGTLVQVFLYSVGRNPAFFRPERYMPQRWLDFG--
A.carolinensis 228 SKVNSLPLLRRAAKETRLRYPVGLFTEFVVSDDIVLQNYHPAGTLVQVFLYSVGRNPAFFRPERYMPQRWLDFG--
X.tropicalis -----
A.schlegelli 243 QKALQGAPELLKGTNKEILRLYPVGTTRQYPIKDIVLQNYHPAGTMVQACLYPGRSAEFEDFELRHEGRWGTSEEG
D.labrax 401 QKALQGAPELLKGTNKEILRLYPVGTTRQYPIKDIVLQNYHPAGTMVQACLYPGRSAEFEDFELRHEGRWGTSEEG
P.notatus -----

H.sapiens 11bH2 434 ---SGRNFHHAFGFGVRQCGRLABAEMLLLHHLHSLVETLTQEDKMYVSFELRESMFLTFRAIN
M.musculus 11bH2 433 ---RSQHAFGFGVRQCGRLABAEMLLLHHLHSLVETLTQEDKMYVSFELRESMFLTFRAIN
H.sapiens 11bH1 434 ---SGRNFHHAFGFGVRQCGRLABAEMLLLHHLHSLVETLTQEDKMYVSFELRESMFLTFRAIN
M.musculus 11bH1 434 ---RSQHAFGFGVRQCGRLABAEMLLLHHLHSLVETLTQEDKMYVSFELRESMFLTFRAIN
A.carolinensis 301 LGKEDNHKAFGFGVRQCGRLABAEMLLLHHLHSLVETLTQEDKMYVSFELRESMFLTFRAIN
X.tropicalis -----
A.schlegelli -----
D.labrax 481 QRGEGTGRSAFAGFGVRQCGRLABAEMLLLHHLHSLVETLTQEDKMYVSFELRESMFLTFRAIN
P.notatus -----

Table 3.2: Sequence identity chart for all sequences used in the 11βH alignment.

	<i>H.Sapiens</i> 11βH2	<i>M.musculus</i> 11βH2	<i>H.Sapiens</i> 11βH1	<i>M.musculus</i> 11βH1	<i>A.carolinensis</i> 11βH	<i>X.tropicalis</i> 11βH	<i>A.schlegelii</i> 11βH	<i>D.labrax</i> 11βH	<i>P.notatus</i> 11βH
<i>H.Sapiens</i> 11βH2	100	69	93	64	51	53	45	43	41
<i>M.musculus</i> 11βH2	69	100	68	80	51	54	44	42	39
<i>H.Sapiens</i> 11βH1	93	68	100	63	50	53	45	42	41
<i>M.musculus</i> 11βH1	64	80	63	100	46	45	39	38	34
<i>A.carolinensis</i> 11βH	51	51	50	46	100	70	50	52	45
<i>X.tropicalis</i> 11βH	53	54	53	45	70	100	55	53	41
<i>A.schlegelii</i> 11βH	45	44	45	39	50	55	100	89	82
<i>D.labrax</i> 11βH	43	42	42	38	52	53	89	100	81
<i>P.notatus</i> 11βH	41	39	41	34	45	41	82	81	100

to energetic coenzymes (NAD/ NADH) mediating the conversion of specific hormone substrates. The midshipman 11 β HSD sequence we cloned is highly conserved across vertebrates and it starts on the carboxy side of the predicted NAD (H) binding site. The amino and carboxy ends of other 11 β HSD sequences are less conserved across species. The amino acid sequences are more conserved downstream from the cofactor binding site (A-domain – GxxxGxG, human 11 β HSD1 41-47; see Fig. 3.2) toward the catalytic domain (D-domain – YxxxK, human 11 β HSD1 183-187; see Fig. 3.2).

Absolute qPCR

We tested the hypothesis that the behavioral/reproductive tactics in type I and type II males would be paralleled by the differential expression of the steroidogenic genes encoding 11 β HSD and 11 β H, and the downstream effector, AR. Robust and significant differences were found between and within (males) the sexes and within a tissue for each gene. The results are presented in Figure 3.4 and in detail (numbers and statistics) in Tables 3.3-3.5 for, respectively, AR, 11 β HSD and 11 β H. Briefly, variance analysis verified both morph (Welch-ANOVA $p < 0.0001$) and gene (Welch-ANOVA $p < 0.006$) specific differences. These differences were found to be significant for AR and 11 β H with $p \leq 0.02$, and for 11 β HSD with $p \leq 0.008$.

Type II males showed significantly higher levels of AR mRNA expression than that of type I males and females in the vocal hindbrain-spinal cord and liver. Type II males also had significantly higher levels of AR than females in forebrain and type I male in testis. Although type I males showed the highest AR mRNA levels among all morphs in the vocal muscle, the levels were only significantly higher than those of females. Type I males also showed significantly higher AR mRNA levels than females in liver. There were no significant differences across all morphs for the middle CNS region sampled that included midbrain, diencephalon and cerebellum.

Figure 3.4: Normalized mRNA values for each adult midshipman morph are plotted against tissue for AR, 11 β HSD and 11 β H genes. Standard errors are noted. Single and double asterisks indicate significance over, respectively; one and two morphs (see text and Tables 1-3 for details). Note break in Y-axis for 11 β H plot, necessitated by higher values in type II male liver.

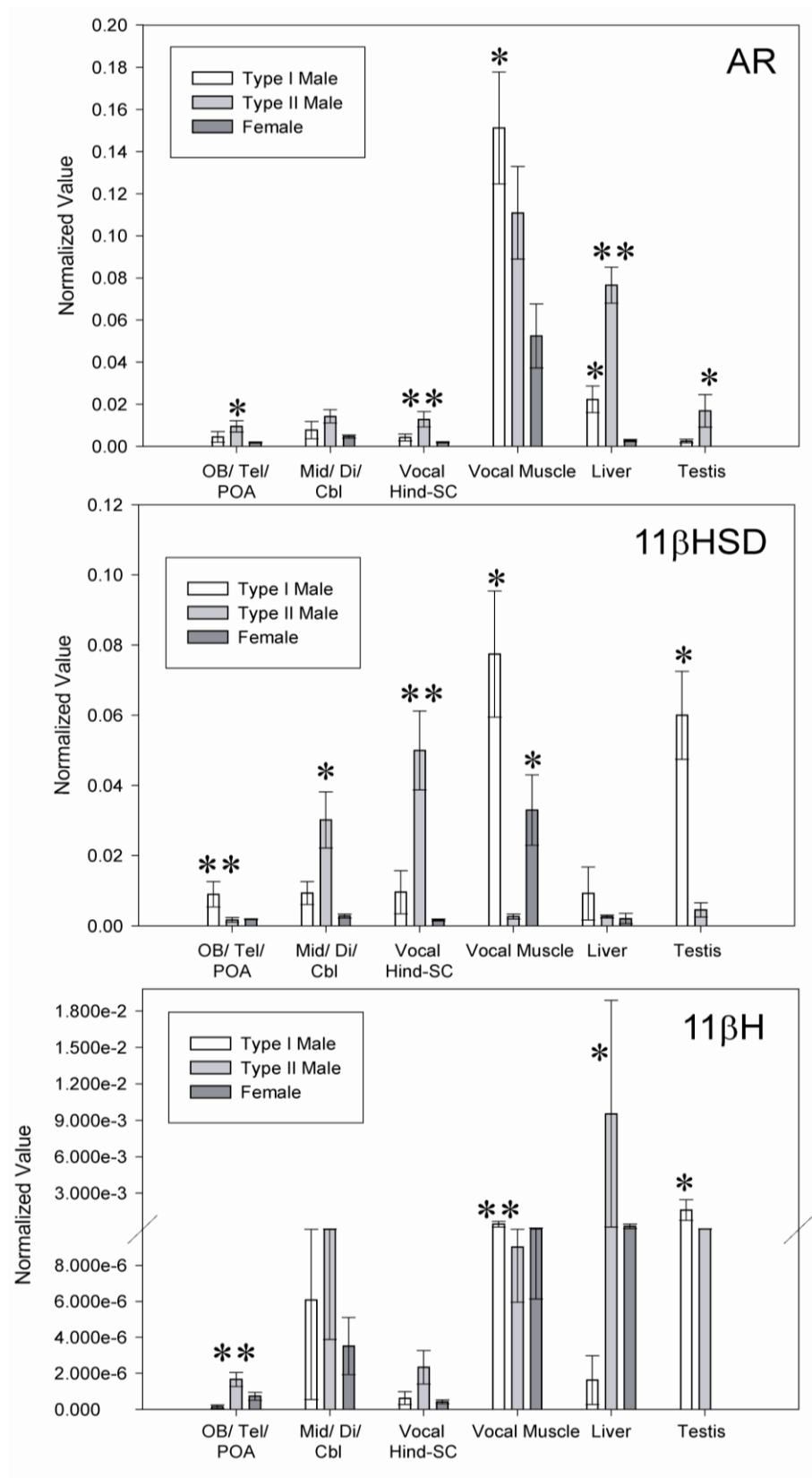


Table 3.3: Normalized values and standard errors for AR mRNA expression are listed according to midshipman morph. ANOVA values (p value, degrees of freedom-dF, and Fratio) are also listed for each analysis performed within each tissue. Footnotes at bottom of the table indicate the direction of significant differences between the morphs.

Morph	Breeding Season Tissue	Norm. mean	Standard error	ANOVA values
Male Type I	OB/ Tel/ POA	0.0044	0.0025	p=0.0109
Male Type II		0.0095 ^{*1}	0.0027	dF=2, 11
Female		0.0018	0.0003	Fratio=7.01
Male Type I	Mid/ Di/ Cbl	0.0076	0.0041	p=0.0957
Male Type II		0.0142	0.0032	dF=2, 12
Female		0.0046	0.0008	Fratio=2.87
Male Type I	Vocal Hindbrain - SC	0.0042	0.0017	p=0.0022
Male Type II		0.0128 ^{*2}	0.0036	dF=2, 12
Female		0.0019	0.0003	Fratio=10.58
Male Type I	Vocal Muscle	0.1512 ^{*3}	0.0266	p=0.0229
Male Type II		0.1109	0.0221	dF=2, 10
Female		0.0524	0.0152	Fratio=5.66
Male Type I	Liver	0.0223 ^{*3}	0.0063	p<0.0001
Male Type II		0.0765 ^{*2}	0.0085	dF=2, 9
Female		0.0027	0.0005	Fratio=35.54
Male Type I	Testis	0.0025	0.0008	p=0.0179
Male Type II		0.0168 ^{*4}	0.0077	dF=1, 6
¹ II > F, ² II > I and F, ³ I > F, ⁴ II > I				Fratio=10.44

Table 3.4: Normalized values and standard errors for 11β HSD values are listed according to midshipman morph. ANOVA values (p value, degrees of freedom-dF, and Fratio) are also listed for each analysis performed within each tissue. Footnotes at bottom of the table indicate the direction of significant differences between the morphs.

Morph	Breeding Season Tissue	Norm. mean	Standard error	ANOVA values
Male Type I Male Type II Female	OB/ Tel/ POA	0.009* ¹ 0.0017 0.0019	0.0036 0.0007 0.0002	p=0.0079 dF=2, 11 Fratio=7.77
Male Type I Male Type II Female	Mid/ Di/ Cbl	0.0093 0.0302* ² 0.0028	0.0033 0.008 0.0006	p=0.0011 dF=2, 11 Fratio=13.63
Male Type I Male Type II Female	Vocal Hindbrain - SC	0.0095 0.05* ³ 0.0016	0.0061 0.0112 0.0003	p=0.0002 dF=2, 11 Fratio=20.29
Male Type I Male Type II Female	Vocal Muscle	0.0774* ⁴ 0.0026 0.033* ⁵	0.018 0.0007 0.01	p<0.0001 dF=2, 12 Fratio=33.48
Male Type I Male Type II Female	Liver	0.0092 0.0026 0.002	0.0075 0.0004 0.0015	p=0.2245 dF=2, 11 Fratio=1.72
Male Type I Male Type II	Testis	0.06* ⁴ 0.0045	0.0125 0.002	p<0.0001 dF=1, 8 Fratio=147.70
¹ I > II and F, ² II > F, ³ II > I and F, ⁴ I > II, ⁵ F > II				

Table 3.5: Normalized values and standard errors for 11bH values are listed according to midshipman morph. ANOVA values (p value, degrees of freedom- dF, and Fratio) are also listed for each analysis performed within each tissue. Footnotes at bottom of the table indicate the direction of significant differences between the morphs.

Morph	Breeding Season Tissue	Norm. mean	Standard error	ANOVA values
Male Type I	OB/ Tel/ POA	1.48x10 ⁻⁰⁷	9.40x10 ⁻⁰⁸	p=0.0023
Male Type II		1.66x10 ⁻⁰⁶ * ¹	3.91x10 ⁻⁰⁷	dF=2, 11
Female		7.26x10 ⁻⁰⁷	2.19x10 ⁻⁰⁷	Fratio=11.06
Male Type I	Mid/ Di/ Cbl	6.08x10 ⁻⁰⁶	5.54x10 ⁻⁰⁶	p=0.7136
Male Type II		2.27x10 ⁻⁰⁵	1.88x10 ⁻⁰⁵	dF=2, 12
Female		3.51x10 ⁻⁰⁶	1.59x10 ⁻⁰⁶	Fratio=0.35
Male Type I	Vocal Hindbrain - SC	6.18x10 ⁻⁰⁷	3.55x10 ⁻⁰⁷	p=0.1112
Male Type II		2.33x10 ⁻⁰⁶	9.30x10 ⁻⁰⁷	dF=2, 10
Female		4.19x10 ⁻⁰⁷	1.08x10 ⁻⁰⁷	Fratio=2.75
Male Type I	Vocal Muscle	3.40x10 ⁻⁰⁴ * ²	2.25x10 ⁻⁰⁴	p=0.0093
Male Type II		9.30x10 ⁻⁰⁶	3.08x10 ⁻⁰⁶	dF=2, 11
Female		6.04x10 ⁻⁰⁵	5.42x10 ⁻⁰⁵	Fratio=7.38
Male Type I	Liver	1.62x10 ⁻⁰⁶	1.35x10 ⁻⁰⁶	p=0.0189
Male Type II		9.54x10 ⁻⁰³ * ³	9.35x10 ⁻⁰³	dF=2, 10
Female		2.62x10 ⁻⁰⁴	1.94x10 ⁻⁰⁴	Fratio=6.05
Male Type I	Testis	1.60x10 ⁻⁰³ * ⁴	8.61x10 ⁻⁰⁴	p=0.0190
Male Type II		4.20x10 ⁻⁰⁵	9.34x10 ⁻⁰⁶	dF=1, 7
¹ II > I and F, ² I > II and F, ³ II > I, ⁴ I > II			Fratio=9.22	

For 11 β HSD mRNA, type II males showed significantly higher levels over type I males and females in vocal hindbrain-spinal cord and over females in the middle CNS region. Type I males showed significantly higher levels over both other morphs in forebrain and over type II males in testis. Both type I males and females expressed significantly higher levels of 11 β HSD mRNA over type II males in the vocal muscle (type I males and females were not different from each other).

For 11 β H mRNA in the forebrain, type II males expressed significantly higher mRNA levels than both type I males and females (type I males and females were not different from each other). There were no significant differences across morphs in the middle CNS region and the vocal hindbrain-spinal cord. Type I males showed significantly higher mRNA levels over type II males and females in vocal muscle (type II males and females were not different from each other), and over type II males in testis.

Although type II males showed significantly higher mRNA expression over type I males alone in liver, the extent of variation for type II males was extreme.

Discussion

This study showed extensive sequence identity between the steroidogenic enzymes 11 β HSD and 11 β H in the plainfin midshipman fish and the same enzymes in other vertebrate groups; a companion study showed the same for the midshipman AR gene (10; P. Forlano, M. Marchaterre, D. Deitcher, A. Bass, to be submitted).

Absolute qPCR then demonstrated significant differences between the two male reproductive morphs (types I and II) in the expression of AR and 11 β HSD mRNA in the CNS and peripheral tissues (vocal muscle, testis, liver); comparable differences were far more modest for 11 β H mRNA. The female expression patterns were distinct from those of both male morphs, although most like that of type I males in the CNS.

Together with a companion study documenting polymorphisms in corticosteroid receptor mRNA levels (Chapter 2), the results show that divergent patterns in androgen and glucocorticoid signaling pathways parallel male morph- and sex-specific, vocal-reproductive phenotypes in midshipman.

Comparisons to mammals

Comparisons of the midshipman AR gene (10) to that in other fish and tetrapods will be presented in detail elsewhere (P. Forlano, M. Marchaterre, D. Deitcher, A. Bass, to be submitted) and thus not discussed here. Two isoforms, 11 β HSD1 and 11 β HSD2, have been reported in mammals and show preferential cofactor and substrate binding that characterizes their divergent metabolic activities (31, 32). The midshipman 11 β HSD sequence is very similar to those in other teleosts, all of which are most like 11 β HSD2 in mammals, especially in the cofactor-binding and catalytic domains (see Fig. 3.2 and Table 3.1) that are conserved among a larger family of enzymes known as short chain alcohol dehydrogenases (32).

The conservation between teleost 11 β HSD and the two mammalian isoforms that function as either a reducing (11 β HSD1) or oxidizing (11 β HSD2) agent suggests that the teleost enzyme may function in both capacities depending on the available substrate. There is considerable conservation in the cofactor-binding domain (human 11 β HSD1 41-47, GxxxGxG) between the teleost 11 β HSD sequences and that of the mammal 11 β HSD2 (see Fig. 3.2 and Table 3.1). The catalytic D-domain (human 11 β HSD1 183-187, YxxxK) of the teleost 11 β HSD seems to be a mix of the domains from the mammalian homologues (Fig. 3.2). The fact that the teleost D-domain contains residues similar to both mammalian homologues suggests that the enzyme may be able to bind either cortisol/11 β -hydroxy (OH)-testosterone (reduction) or cortisone/11KT (oxidation) (11 β -OH testosterone is an intermediary in the conversion

of testosterone to 11KT (3; Fig. 3.1). The latter might enable the teleost 11 β HSD to function either as a C11-dehydrogenase in tissues like testis and thus affect androgen synthesis (discussed further below), but as a C11-hydroxylase in the CNS with a more potent influence on corticosteroid pathways. There is also evidence for the C11-dehydrogenase activity of the teleost 11 β HSD in testis (rainbow trout, Japanese eel, and tilapia) via induction of dihydroprogesterone, a 5 α -reduced metabolite of progesterone (33-36).

Among mammals, 11 β HSD1 and 11 β HSD2 show differing patterns of central and peripheral expression, inclusive of changes during ontogeny (32). Peripherally, the 11 β HSD2 isoform that most closely resembles 11 β HSD in teleosts is abundant in mammalian kidney and placenta (32). Activity levels for the 11 β HSD2 enzyme are several fold higher in the hindbrain than the forebrain at several neonatal timepoints (37), comparable to the hindbrain - forebrain divergence in 11 β HSD mRNA levels in type II male midshipman (Fig. 3.4). Elevated 11 β HSD2 mRNA early in development likely acts to protect the CNS from the potential deleterious effects of glucocorticoids (see above references); a similar function is proposed for midshipman (see below).

The midshipman 11 β H enzyme is most similar to the mammalian 11 β H type 1 isoform that converts 11-deoxycortisol to cortisol in humans and 11-deoxycorticosterone to corticosterone in rodents (38). It has been proposed that the CYP11B gene underwent a duplication event among ancestral mammals, yielding CYP11B1 and CYP11B2 that encode, respectively, 11 β H and aldosterone synthase (converts 11-deoxycorticosterone to aldosterone) (39, 40). The distribution of 11 β H mRNA throughout the body has not been well studied in mammals; levels are generally highest in the adrenal with more variable profiles between the brain and other tissues (41, 42).

Male morph divergence in midshipman fish

We first provide an extended discussion of the dimorphic patterns in mRNA expression for each tissue studied here before turning to a summary commentary. We recognize the potential limits of using beta actin (BA) as the single reference gene for qPCR. Initial cloning investigated both BA and glyceraldehyde 3-phosphate dehydrogenase (GADPH), but GADPH primers did not perform consistently. However, BA variation was less than 5% between morphs within a given tissue and worked well in this system (data not shown). In addition, variation in BA between tissues was much greater than 5% and for this reason we did not compare expression between peripheral and central tissues. Beta actin expression has been suggested to differ between reproductive (R) and non-reproductive (NR) male morphs in a cichlid (43). Hence, BA may yet be regulated in a manner not apparent in the comparisons made for the current study of midshipman, although R and NR males are not comparable to type I and II midshipman, both of whom are reproductively active (44).

Testis

As reviewed in the Introduction, midshipman fish show male morph- and sex-specific profiles in circulating steroids. Although proposed earlier for teleosts (e.g., 45), the results presented here show the predominant expression of 11 β HSD, the one enzyme essential for the final conversion of testosterone to 11KT (Fig. 3.1), in the testis of the displaying male morph (type I male midshipman) in a species with alternative reproductive tactics. The low, but elevated 11 β H gene levels in type I males compared to the other morphs is also consistent with the detection in some type I males of 11 β -OH-testosterone (18), an intermediate in the conversion of testosterone to 11KT (Fig. 3.1). Given the co-elevation of 11 β HSD gene levels in the testis and 11KT in the plasma of type I males, together with the reported absence of C21-

hydroxylase in fish testis that leads to glucocorticoid synthesis (6), the 11β HSD and 11β H genes likely serve as dimorphic regulators of plasma androgen levels in the plainfin midshipman and other teleosts that display alternative male reproductive tactics.

The divergent patterns between male morphs in AR levels in the midshipman testis suggest that it could be a regulatory factor leading to the divergence between the male morphs in testis function, inclusive of 11KT production, between the two male morphs. Consistent with the role of androgenic mediation of testicular growth/retardation (2, 46), type II males express more AR and exhibit relatively larger testis for their body size than type I males (14, 44).

The capacity for interaction between androgenic components may influence the functional state of the testis (35, 47, 48). Hence, male morph differences in AR, 11β HSD and 11β H mRNA expression levels may together contribute to mechanisms underlying higher sperm motility in type II males (49, 50).

Vocal muscle

The AR studied here was originally isolated from midshipman vocal muscle (10). Except for the liver, AR has its highest expression levels in the vocal muscle of type I males, consistent with the androgen-dependent hypertrophy of vocal muscle in juvenile males (18, 20). While reduced in comparison to type I males, the robust expression of AR in the vocal muscle of type II males and to a lesser degree in females, concurs with the androgen-induced increases in vocal muscle mass in both of these morphs as well (20).

The androgen-dependency of vocal muscle morphology is consistent with other results showing that the effect is reversible following the cessation of androgen treatments in juvenile males and females (20).

Like AR, the 11 β HSD gene showed the most robust expression in the vocal muscle of type I males; though far lower, 11 β H gene expression was similarly elevated in comparison to other morphs. The maintenance of a larger vocal muscle mass in type I males during the breeding season (51) may be dependent on 11KT available from both the testis via the circulation and local synthesis via 11 β HSD action; seasonal elevation of AR may also help support a larger vocal muscle.

Somewhat surprisingly, 11 β HSD levels were much higher in the vocal muscle of females than type II males despite their similar vocal repertoires, namely the production of brief grunts (44). Vocal behavior in females and type II males has, however, been little studied in comparison to type I males (44, 52). Females may yet be far more vocal than type II males during the breeding season, dependent in part on local 11KT synthesis in the vocal muscle. Elevated 11 β HSD levels in the female muscle might also act as a sink for lowering circulating cortisol levels. As discussed below, reduced vocal behavior in type II males would be consistent with a sneak-spawning tactic.

Given 11 β HSD's role in cortisol synthesis in teleosts (Fig. 3.1), it may be serving both an androgenic and glucocorticoid function in the vocal muscle of all morphs. However, compared to type II males and females, type I males show about three-fold higher levels of glucocorticoid receptors in the vocal muscle (Chapter 2). The robust co-elevation of corticosteroid and androgen mediated gene-regulatory mechanisms likely aids substantially in the gluconeogenic and metabolic demand of the type I male vocal muscle during the breeding season, especially the type I male's production of advertisement hums that may last > 1h (see Introduction).

Liver

The low expression of steroidogenic enzymes in the liver of all morphs may indicate that the liver, in general, contributes little to circulating levels of steroids. Androgen receptor mRNA expression showed prominent divergence between the midshipman morphs. Type II males had the highest AR mRNA levels, although type I males show the highest glucocorticoid receptor mRNA levels in the liver (Chapter 2). Elevated AR levels together with significantly higher plasma cortisol levels in type II males (Chapter 2), may place limits on glucocorticoid receptor expression in the liver. However, this relationship may be tissue specific; although AR mRNA levels in the testis are higher in type II males, glucocorticoid receptor mRNA levels are about equal between the two male morphs (Chapter 2). Androgen receptors are localized to hepatocytes in mammalian liver (53). Hence, the elevation of AR mRNA in type II male liver may relate to divergent patterns of hepatocyte function that is essential to the formation and secretion of bile.

Central nervous system (CNS)

Given that 11KT is the predominant androgen in type I males (see Introduction) and the highest levels of 11 β HSD mRNA and 11 β H mRNA are in the testis and vocal muscles of type I males, one might expect a similar morph-specific pattern for the CNS. Somewhat surprisingly, CNS expression levels for both the 11 β HSD and AR genes are, in general, the highest in type II males (11 β H was essentially low to undetectable in all morphs). The AR results are consistent with *in situ* hybridization studies showing abundant AR in localized regions of the forebrain, midbrain and the vocal hindbrain-spinal region (10). Also like the qPCR results reported here, preliminary *in situ* mapping indicates that AR is most abundant in the CNS of type II males (P. Forlano, M. A. Marchaterre, A. Bass, unpublished

observations).

The divergence in AR abundance between the midshipman male morphs is also consistent with the predominant effects of either testosterone or 11KT on vocal motor patterning in, respectively, type II and type I males (12, 22). Testosterone modulation of the female vocal system resembles that shown for type II males. However, receptor and enzyme blocking experiments show that the female response depends on an estrogen receptor mechanism (12), consistent with the lowest AR levels in the vocal hindbrain-spinal cord of females. Although AR levels were several fold lower in the vocal hindbrain-spinal cord of type I males, AR could still support the 11KT modulation of vocal patterning in this morph (22). Midshipman have one form of AR, the AR subtype studied here (10; P. Forlano, M. Marchaterre, D. Deitcher, A. Bass, to be submitted). Studies of the one AR identified in zebrafish (54, 55), which is similar in sequence to the midshipman AR (P. Forlano, M. Marchaterre, D. Deitcher, A. Bass, to be submitted), show that 11KT is a more potent activator of the AR than testosterone (55), consistent with 11KT's more potent induction of vocal muscle hypertrophy in midshipman (18). Hence, despite lower AR levels in type I male CNS, AR may also mediate 11KT actions on vocal function due to 11KT's potent activation of the AR receptor.

In regards to 11 β HSD, given that type II males have the highest CNS levels of glucocorticoid receptor mRNA (Chapter 2), undetectable levels of plasma 11KT (15) and two fold higher levels of plasma cortisol compared to type I males (13), the most parsimonious interpretation is that the high 11 β HSD mRNA levels in type II males are related to the conversion of cortisol to an inactive metabolite, cortisone (see Fig. 3.1). The abundance of 11 β HSD mRNA in the hindbrain-spinal region, together with the known effects of cortisol on the vocal motor output of type II males (12), would predict localization to the vocal pattern generator that is expansive in this region (24).

The cortisol-dependent suppression of the vocal motor system in type II males (12) might depend, under natural conditions during sneak-spawning, on chronically higher cortisol levels in type II males (13). Locally elevated 11 β HSD levels could then provide for the rapid conversion of cortisol to an inactive metabolite (cortisone) to protect the vocal pattern generator from potential cortisol-related neurotoxicity (56). Alternatively, cortisone might be a neuroactive compound in this system and future neurophysiological studies should investigate this hypothesis. Support for cortisol-dependent suppression of natural vocalization in this family of fish (Batrachoididae) comes from field studies of the closely related toadfish, *Opsanus beta* that show rapid (within mins), concurrent decreases in calling behavior and increases in plasma cortisol levels in males following underwater playbacks of sounds that mimic the calls of predatory dolphins (57).

Forebrain levels of 11 β HSD mRNA were highest in type I males, unlike the rest of the CNS where they were highest in type II males. The forebrain divergence might relate to the role of the preoptic area (POA), that is rich in glucocorticoid receptors (58), in a positive feedback mechanism supporting the 2-3 fold higher glucocorticoid (cortisol) levels in type II males (Chapter 2). Concurrent elevation of plasma cortisol and glucocorticoid receptor mRNA in peripheral tissues has been reported in other teleosts (59, 60). A positive feedback mechanism in midshipman forebrain would be consistent with type II males having the highest expression of glucocorticoid receptor in the POA (Chapter 2) and higher cortisol levels available from both the circulation and local synthesis in the POA (via high 11 β H and low 11 β HSD; see Figs. 3.1, 3.4). By contrast, comparatively reduced glucocorticoid receptor levels in the POA of type I males (Chapter 2) would be consistent with their lower cortisol levels in both the circulation and locally within the POA (dependent, in part, on low 11 β H and/or elevated 11 β HSD; see Figs. 3.1, 3.4). Future studies need to

test these hypotheses using quantitative *in situ* hybridization methods to assess glucocorticoid receptor mRNA levels in the POA.

The generally greater expression of 11 β HSD mRNA in the CNS of both male morphs compared to females suggests that 11 β HSD figures more prominently into the expression of male morph- rather than sex-specific behaviors. A glucocorticoid suite of characters further distinguishes the two male morphs. Type II males show the highest levels of plasma cortisol and CNS mRNA for both 11 β HSD and glucocorticoid receptors that may reflect their sneak-spawning tactic that requires them to be on “flight” alert at all times. Type I males are more typically in a “fight” rather than a “flight” mode during nest occupancy; type II males are frequently attacked and escaping from the aggressions of nest-guarding type I males (44). As discussed above, high 11 β HSD levels in type II males may support ongoing vocal inhibition, consistent with a tactic to minimize acoustic detection by type I males. One caveat here is that cortisol also suppresses vocal motor output in females (12), although females have the lowest 11 β HSD mRNA levels and relatively low glucocorticoid receptor mRNA levels throughout the CNS. However, nesting females like the ones sampled in this study (see Materials and Methods) may not suppress vocalization during spawning. Low amplitude grunts, typical of the vocal signals of females recorded in non-spawning contexts, have been detected during type I male-female interactions in the nest when type II males were not present (44).

Functional overview of male morph-specific expression patterns

Differential gene expression between male morphs in a single tissue type such as the testis leads to obvious conclusions regarding steroid biosynthesis and male morph-specific profiles of plasma steroids in midshipman fish (see Introduction and earlier discussion of testis). Similarly, a focus on the sound-producing, vocal muscle

leads to clear conclusions given its essential role in vocalization and sexual polymorphisms in midshipman (see Introduction and earlier discussion of vocal muscle). By contrast, studies of entire CNS regions inherently constrain functional interpretations. However, studies of the midshipman's hindbrain-spinal cord region have led to cogent hypotheses regarding the functional significance of divergent patterns of mRNA and protein abundance. The hindbrain-spinal cord region contains the expansive vocal pattern generator circuit that directly establishes the temporal properties of vocal behavior (24, 61). The initial demonstration of elevated aromatase activity levels in this CNS region predicted localization of aromatase mRNA and protein to the vocal system (25). Subsequent neuroanatomical studies supported this hypothesis (29, 62), while neurophysiology showed that blocking aromatase action could interfere with testosterone-dependent modulation of vocal motor patterning (12). Neurophysiological studies also demonstrated androgen modulation of the vocal system (12, 22), supported by *in situ* hybridization showing AR mRNA abundance in vocal premotor and motor nuclei in the hindbrain-spinal region (10; P. Forlano, M. Marhcaterre, D. Deitcher, A. Bass, to be submitted). Similarly, we predict the localization of steroidogenic enzyme (11 β HSD, 11 β H) and glucocorticoid receptor mRNAs to the vocal system given the salient effects of androgens and cortisol on vocal neurophysiology (12, 22).

Given the interpretive strength offered by studies of steroid-sensitive vocal phenotypes in midshipman fish (above), we compare the genomic profiles identified here, along with those for the glucocorticoid receptor (GR) (Chapter 2), for the vocal hindbrain-spinal region, the vocal muscle, and the testis. For simplicity, we limit the comparisons to the midshipman's two male morphs. In general, the female expression pattern is distinct in that CNS mRNA levels are uniformly the lowest among all adult morphs, while the vocal muscle profiles are more variable (Fig. 3.4, also see earlier

sections of the discussion regarding females). Table 3.6 shows the direction and relative magnitude (expressed as fold differences) of AR, 11 β HSD, 11 β H and GR mRNA levels for type I and type II male midshipman. The following patterns emerge:

1. Vocal hindbrain-spinal cord: 11 β HSD, AR and GR mRNA levels are higher in type II males. As discussed in detail in the previous section, the higher 11 β HSD levels could play a powerful role in modulating local levels of cortisol that suppress (via higher GR) vocal motor output in type II males alone (12, 22). The higher AR mRNA levels would support the testosterone/ androgen receptor-specific augmentation of vocal motor output in type II males (12).
2. Vocal Muscle: All four genes show higher expression in type I males. The dramatically elevated 25-40 fold higher levels of both 11 β HSD and 11 β H mRNAs could provide locally high 11KT synthesis (Fig. 3.1). The latter could supplement 11KT available from the circulation to augment 11KT's actions (via AR) on the expression of the type I male-specific muscle morphology and profile of oxidative enzymes (14, 15, 18, 20, 63). Higher 11 β HSD mRNA levels could also modulate local cortisol levels, as proposed above for the vocal hindbrain. In this case, cortisol action, acting via elevated GR, may play an essential role in support of the energetic demands of long duration advertisement (hums) and agonistic (grunt trains) vocalization by type I males alone (44, 52).
3. Testis: The 12-40 fold higher levels of 11 β HSD and 11 β H mRNA in type I males is consistent with local 11KT synthesis (see Fig. 3.1) underlying the elevated plasma 11KT levels found in this morph alone (7, 18, 64). Morph-specific profiles of 11 β HSD/ 11 β H (higher in type I males) and AR (five fold higher in type II males) mRNA expression could also influence differences in sperm motility between the male morphs (49).

Table 3.6: Summary of fold differences (x) between type I and type II male midshipman fish in the mean mRNA values for AR, 11 β HSD and 11 β H studied here (see Fig. 3.4, Tables 3.3-3.5) and the glucocorticoid receptor (GR) in the companion study (Chapter 2).

	AR	11βHSD	11βH	GR
Vocal Hindbrain - SC	II 3X > I	II 5X > I	-	II 6X > I
Vocal Muscle	I 0.4X > II	I 26X > II	I 37X > II	I 2.5X > II
Testis	II 5X > I	I 12X > II	I 40X > II	-

Table 3.7: Summary of hormonal and gene morph-specific trends.

	Hormones	VH-SC	Vocal Muscle	Testis
Type I male	↓ Cortisol	↓ 11 β HSD	↑ 11 β HSD, 11 β H	↑ 11 β HSD, 11 β H
	↑ 11KT	↓ AR, GR	↑ AR, GR	↓ AR
Type II male	↑ Cortisol	↑ 11 β HSD	↓ 11 β HSD, 11 β H	↓ 11 β HSD, 11 β H
	↓ 11KT	↑ AR, GR	↓ AR, GR	↑ AR

Collectively, the results summarized in Table 3.6 highlight the distinction between the vocal-endocrine/ reproductive phenotypes of the two male morphs in midshipman fish (see Introduction and above for references, also see Table 3.7). Unlike type I males, type II males neither acoustically court females with advertisement calls nor defend nests with agonistic calls. Type II males are only known to either sneak or satellite spawn. Only type II males exhibit testosterone-sensitive, vocal motor patterning via an androgen receptor mechanism. While cortisol suppresses vocal motor output in type II males, it facilitates output in type I males. The divergent expression patterns observed here reflect the distinct profile of circulating steroids for each male morph, as shown in neurophysiological studies of differential steroid effects on vocal function (12). Compared to type I males, type II males show significantly higher plasma levels of testosterone and cortisol with undetectable 11KT; 11KT levels are up to 10 fold higher than testosterone in type I males.

The current study now sets the stage for investigating morph divergence in levels of steroids along with steroid receptors and biosynthetic enzymes in localized CNS regions (e.g., vocal hindbrain-spinal cord) and peripheral tissues (e.g., vocal muscle) during the performance of morph-specific vocal and reproductive behaviors (see 65).

Concluding comments

Steroidogenic receptors and enzymes have played critical roles in the evolution of the reproductive and stress axes (46, 66, 67). This new study, together with a companion one (13), provides an initial assessment of divergent patterns in the expression of genes encoding steroid receptors and the enzymes regulating both

androgen and glucocorticoid synthesis in a teleost fish with alternative male reproductive morphs. As discussed above, the divergence in enzymatic patterning and steroid receptor expression between the two male morphs in midshipman fish are likely fundamental to the role of steroids in maintaining morph-specific, vocal tactics among reproductively-active adults. While not discussed, the divergent gene expression patterns identified here may also contribute to the adoption of one of the two alternative male developmental trajectories during pre-adult, life history stages (16).

The distinct genomic profiles reported here for types I and II males further show that species with alternative male reproductive tactics offer unique opportunities to identify the mechanistic basis of the divergent neuroendocrine phenotypes observed among both fish and tetrapods (4, 8, 62, 68). Among humans, stress and reproductive regulation via, respectively, glucocorticoid- and androgen-related proteins are important factors influencing both mental and physical health (69-71). Comparative approaches to the interaction of such axes can now begin to provide a more basic, molecular and evolutionarily-based foundation showing how systemic hormonal properties contribute to both adaptive and maladaptive mechanisms of behavioral modulation via steroid signaling mechanisms.

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CHAPTER 4

THE EVOLUTION OF STEROID SPECIFICITY IN DUPLICATED CORTICOSTEROID RECEPTOR GENES AMONG VERTEBRATES

Abstract

The evolution of steroid receptor genes across vertebrates has often been interpreted within the context of the conservation and divergence of their protein sequences. The presence of a mineralocorticoid (MR) and duplicate glucocorticoid (GR) receptors in teleost fish has been an area of much investigation because of remaining uncertainties in how they compare functionally to the MR and GR of tetrapods. Prior studies show that the corticosteroid receptors of teleosts, like those of mammals, can be distinguished by their relative sensitivity to aldosterone and/or cortisol. Using a luciferase-based reporter gene assay, we compared the specificity of the ligand binding domain (LBD) of corticosteroid receptors from two distantly related groups of teleosts, cichlids and midshipman fish, to the MR and GR of a mammal (mouse). Both cichlids and midshipman had a receptor like the mouse GR that was activated by cortisol and the synthetic glucocorticoid dexamethasone, but not by the mineralocorticoid aldosterone. A duplicated receptor in cichlids, known as GR2, showed high sequence identity to the mouse GR, but responded like the mouse MR to aldosterone, cortisol, and dexamethasone. A phylogenetic analysis suggested that the cichlid GR2 secondarily evolved after a more ancestral duplication event that led to the initial divergence of two corticosteroid receptors that were activated by either cortisol alone (GR) or both cortisol and aldosterone (MR). Further analysis also

showed that GR2's MR-like phenotype may have resulted from single residue changes. Thus, alterations in the specific residues within the protein sequence of corticosteroid receptors may lead to divergent receptor-ligand specificity that has evolved multiple times throughout vertebrate evolution.

Introduction

The nuclear receptor super-family of proteins includes steroid receptors that are ligand-activated transcription factors mediating steroid actions on a multitude of behavioral, morphological and physiological processes (1). The two major functional groups of vertebrate corticosteroid receptors among tetrapods are the glucocorticoid (GR) and mineralocorticoid (MR) receptors, which have distinct amino acid sequences and ligand specificity. The transcriptional activities of the GR and MR are induced by cortisol, the principal glucocorticoid among humans and many other vertebrates, and corticosterone, the major glucocorticoid among many tetrapods (1). The MR is distinguished by a ten-fold greater sensitivity to cortisol and is also activated by the mineralocorticoid aldosterone (1, 2).

There are two major groups of living fishes, the Actinopterygii which include teleosts (the largest group of living vertebrates) and the Sarcopterygii which include lungfish (Dipnoi) and the coelacanth (Latimeria), and gave rise to tetrapods (3). Lungfish, like tetrapods, have detectable circulating levels of cortisol and aldosterone (1). Among teleosts, like humans, cortisol is the major glucocorticoid (1), but there is no convincing evidence for significant circulating levels of aldosterone in teleosts (4, 5). Several teleosts also have multiple corticosteroid receptors that have been classified as either a GR or MR depending on their sequence identity to the mammalian receptors and their ligand sensitivity (6, 7). The transcriptional activation

of some teleost corticosteroid receptors is induced *in vitro* by aldosterone (8-11). The existence of teleost receptors with aldosterone sensitivity, but where cortisol may be the predominant ligand in the absence of aldosterone, has prompted investigations like the current one into the functional relationship and evolution of corticosteroid receptors and the ligands that induce their transcriptional activation. The current study specifically focused on comparing the ligand specificity of the ligand binding domain (LBD or E domain) of corticosteroid receptors between two distantly related teleosts (cichlids/ Perciformes and toadfishes/ Batrachoidiformes, (3) and a mammal (mouse). The cDNA encoding the LBD was first cloned and then linked to the coding sequence for the DNA binding domain (DBD) of the yeast transcription factor GAL4. The transcriptional activation of each of GAL4-LBD fusions was then determined in response to corticosteroids using a cell based reporter gene assay. Since multiple domains of steroid receptor gene products can influence transcriptional activation (12-15), we used a minimal promoter / UAS system identical to the GAL4/UAS system employed *in vivo* in *Drosophila melanogaster* which allowed extremely low levels of basal transcriptional activity (11). This approach thus enabled a specific assessment of the evolutionary relationship between sequence identity and ligand specificity of the LBD within this family of proteins.

As reported, a duplicated corticosteroid receptor in a teleost fish with a sequence identity closest to the mammalian GR, is activated by both cortisol and aldosterone like the mammalian MR. The major determinants of ligand specificity appear to lie at the single residue level within the LBD. A phylogenetic analysis of the protein sequences of corticosteroid receptors established the relationships of the aldosterone-sensitive and insensitive receptors of teleosts to those of other vertebrates. Together, the results provide new insights into the molecular events that have led to the evolutionary divergence of corticosteroid receptors via genome modification

events.

Materials and Methods

Tissue sources

Midshipman fish (*Porichthys notatus*) were hand collected from field sites in northern California during the summer breeding season (May-August), shipped to Cornell University within 72h, and maintained in seawater tanks until sacrificed for tissue within 24-48h of receipt. Tissue sampling (liver) was carried out following deep anesthetization (0.025% benzocaine; Sigma, St. Louis, MO). All experimental protocols were approved by the Cornell University Institutional Animal Care and Use Committee. Mouse liver (*Mus musculus*) was kindly donated by David McCobb (Department of Neurobiology and Behavior, Cornell University) and cichlid liver (the Daffodil cichlid, *Neolamprologus pulcher*) by Sigal Balshine (Department of Psychology, McMaster University). Cichlids were chosen because duplicate GRs (GR1, GR2) and a single MR have been identified (9). Midshipman fish were chosen, in part, because they are a distantly related teleost (3) and appear to only have one GR (33). All liver samples were flash frozen in liquid nitrogen and stored at -80°C until use for RNA extraction.

Cloning of the receptor LBDs

RNA was isolated from the liver of each species (*M. musculus*, *P. notatus*, *N. pulcher*) using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. PCR on cDNA from all tissues were conducted using gene/species-specific primers that targeted the LBD of each reported corticosteroid receptor: mouse GR, mouse MR, cichlid MR, cichlid GR1, cichlid GR2, and

midshipman GR (see Fig. 4.1 and Table 4.1 for sequence information). The midshipman MR was not included because the reported sequence is only a part of the LBD and, like the MR of other teleosts, shows very high sequence identity to the cichlid MR and that of other teleosts at the level of the LBD (33). Therefore, the cichlid MR ligand specificity is considered to be representative of the MR of midshipman and other teleosts. The primers were designed to amplify a highly conserved region based on an alignment of vertebrate corticosteroid receptors (sequences are presented in Fig. 4.1). Amplification was performed using *Taq* polymerase (Epicentre) under the following cycles: Stage 1 – 94°C for 2 min (1 cycle), Stage 2 – 94°C for 1 min, 52°C for 1 min, 72°C for 3 min (35 cycles), and Stage 3 – 72°C extension for 10 min (1 cycle), and resulted in the predicted product size for each LBD (see Table 4.1 for product sizes). Products were cloned into the Bluescript KS (+) plasmid, subcloned using DH5 α competent cells, and plated. Individual colonies were grown in liquid culture, mini-prepped (Qiagen, Germantown, MD), and sequenced at the Cornell University Life Sciences Core Laboratory Center using T3 and T7 primers.

Construction of GAL4 fusions and a GAL4-driven luciferase reporter

The GAL4 DNA binding domain (aa 1-149), engineered with a FLAG-tagged epitope tag (DYKDDDDK) at its amino terminus, was amplified and cloned into the Bluescript KS (+) plasmid, subcloned using DH5 α competent cells, and plated. Individual colonies were grown in liquid culture, mini-prepped (Qiagen, Germantown, MD), and sequenced at the Cornell University Life Sciences Core Laboratory Center using T3 and T7 primers. The LBD was then subcloned into the GAL4 (DBD)-Bluescript KS(+) plasmid, and used to transform competent cells. The GAL4 DBD –

Figure 4.1: An alignment of the GR and MR LBD fragments used in the steroid transactivation assays. In addition, skate (*L. erinacea*) GR and MR LBD fragments have been included as a comparative control, as it represents a primitive GR with aldosterone sensitivity (see Fig. 4). Asterisks above the top row indicate residues important for C18 carbonyl versus C17 hydroxyl binding. Open circles indicate residues important for C18/C17 binding that are conserved within MRs (above the top row) and GRs (below bottom row). A plus symbol represents residues that are changed within all teleost GRs compared to mammalian GRs and therefore not likely to influence the C18 specificity of *N. pulcher* GR2. Black-filled circles represent known binding sites of functional groups (carbonyl versus hydroxyl) for both glucocorticoids and mineralocorticoids.

<i>L. erinacea</i> MR	1	LQGYVPEKMSRSPMLDRSCSSVTTTPSKVCLVCADEASGCHYGVLTGSCVKVFVKRAVEGQQNYLCAGRNDCIIDKIR
<i>N. pulcher</i> MR	628	-----GVTTCGSCKVFLKRAVEGQHNYLCAGRNDCIIDKIR
<i>P. notatus</i> MR	1	-----
<i>M. musculus</i> MR	661	-----
<i>L. erinacea</i> GR	1	-----RNDCIIDKIR
<i>N. pulcher</i> GR	495	-----IR
<i>N. pulcher</i> GR1	470	-----RR
<i>P. notatus</i> GR	1	-----
<i>M. musculus</i> GR	500	-----
* End of predicted DBD Start of predicted LBD *		
<i>L. erinacea</i> MR	81	KNCPACRLRKWLAGMNLGARKSKKCKVKGHEEQ-----QPPHSPKEGIMFASLPEPGSTTLTF-HFSLMPPHVS
<i>N. pulcher</i> MR	665	KNCPACRVRKCLQAGMNLGARKSKKCKVKGSEGLQGSKDGQTATGGAGGYLSSSEKELNAANALPHRPGVVTFTLP
<i>P. notatus</i> MR	1	-----PYL
<i>M. musculus</i> MR	661	-----OKCLQAGMNLGARKSKKCKVKGHEEQPPPPPPPPPEEGTYIAPTKEPVNSALTF-GLASITRALTF
<i>L. erinacea</i> GR	12	KNCPACRFKCKLQAGMNLGARKKKCKVKG-----HSSSTSETTKAVSQD-LTLVRPSLTF-----VLS
<i>N. pulcher</i> GR2	498	KNCPACRFKCKLQAGMNLGARKKKCKVKG-----RAGAPEFTISNMFVP-----VVPRLSLTF-----QLV
<i>N. pulcher</i> GR1	472	KNCPACRFKCKLQAGMNLGARKKKCKVKG-----QSSNPPEVTSPPVETRLSLVPCMLTF-----QLV
<i>P. notatus</i> GR	1	-----PACRFKCKLQAGMNLGARKKKCKVKG-----RSSETSESSISLPPP-----FTPRALSLTF-----QLV
<i>M. musculus</i> GR	500	-----AGMNLGARKKKCKVKG-----QQATAGVSDTSENANKTLVPAALSLTF-----QLV
<i>L. erinacea</i> MR	154	PSLTITLBAIEPEVYASYDSSQADTNYLLSLENRLAEKQMRIVKWKAGLPGFRITVALDDQMTLLRYSWMCLMFSIS
<i>N. pulcher</i> MR	744	PSLCSMLEIEPEVYSGYDSSQPDTHLLSLENRLAGQMVRVWKAVLPGFRSLPIEDQTLTQYSWMCLSSFCIS
<i>P. notatus</i> MR	5	PSLCSMLEIEPEVYSGYDSSQPDTHLLSLENRLAGQMVRVWKAVLPGFRSLPIEDQTLTQYSWMCLSSFCIS
<i>M. musculus</i> MR	733	PSPSMLEIEPEVYAGYDSSQPDTHLLSLENRLAGQMIVKWKAVLPGFRSLPIEDQTLTQYSWMCLSSFCIS
<i>L. erinacea</i> GR	69	PSVTSLEMLEIEPDLIYAGYDSSQPDTPNRLSLGLNGLQMTSVVWKARTLPGFRSLHLLDDQMTLLQYSWMCLMFSIS
<i>N. pulcher</i> GR2	555	PTMLSILKAIEPEITLYSGYDSTTPDTSRLMTLNLRLGGQVSAVKWAKSLPGFRSLHLLDDQMTLLQYSWMCLMFSIS
<i>N. pulcher</i> GR1	529	PTMLSILKAIEPEITLYSGYDSTTPDTSRLMTLNLRLGGQVSAVKWAKSLPGFRSLHLLDDQMTLLQYSWMCLMFSIS
<i>P. notatus</i> GR	55	PTMLSILKAIEPEITLYSGYDSTTPDTSRLMTLNLRLGGQVSAVKWAKSLPGFRSLHLLDDQMTLLQYSWMCLMFSIS
<i>M. musculus</i> GR	545	PTLVSILEVIEPEVYAGYDSSQPDPAWRMLMTLNLRLGGQVSAVKWAKSLPGFRSLHLLDDQMTLLQYSWMCLMFSIS
<i>L. erinacea</i> MR	234	WRSYKHNGRMLFAPDLVNEERMORSAMYLCQGMOSTSIEFVRLQLTQEEFLCMKVLLLLSTVPKDGKLSQAFDEI
<i>N. pulcher</i> MR	824	WRSYKHNGRMLFAPDLVNEERMORSAMYLCQGMOSTSIEFVRLQLTQEEFLCMKVLLLLSTVPKDGKLSQAFDEI
<i>P. notatus</i> MR	85	WRSYKHNGRMLFAPDLVNEERMORSAMYLCQGMOSTSIEFVRLQLTQEEFLCMKVLLLLSTVPKDGKLSQAFDEI
<i>M. musculus</i> MR	813	WRSYKHNGRMLFAPDLVNEERMORSAMYLCQGMOSTSIEFVRLQLTQEEFLCMKVLLLLSTVPKDGKLSQAFDEI
<i>L. erinacea</i> GR	149	WRSYKHNSQMLFAPDLVNEERMORSAMYLCQGMOSTSIEFVRLQLTQEEFLCMKVLLLLSTVPKDGKLSQAFDEI
<i>N. pulcher</i> GR2	635	WRSYQCCNGSMILCFAPDLVNEERMORSAMYLCQGMOSTSIEFVRLQLTQEEFLCMKVLLLLSTVPKDGKLSQAFDEI
<i>N. pulcher</i> GR1	609	WRSYQCCNGSMILCFAPDLVNEERMORSAMYLCQGMOSTSIEFVRLQLTQEEFLCMKVLLLLSTVPKDGKLSQAFDEI
<i>P. notatus</i> GR	135	WRSYQCCNGSMILCFAPDLVNEERMORSAMYLCQGMOSTSIEFVRLQLTQEEFLCMKVLLLLSTVPKDGKLSQAFDEI
<i>M. musculus</i> GR	625	WRSYQCCNGSMILCFAPDLVNEERMORSAMYLCQGMOSTSIEFVRLQLTQEEFLCMKVLLLLSTVPKDGKLSQAFDEI
<i>L. erinacea</i> MR	314	RMNYIKELRRTVIAENENNTGONWQRFYQLTKLDSMHDLVGLLQICFYTFNKSALKEFFMLVEIINDQFEVTTGM
<i>N. pulcher</i> MR	904	RMNYIKELRRTVIAENENNTGONWQRFYQLTKLDSMHDLVGLLQICFYTFNKSALKEFFMLVEIINDQFEVTTGM
<i>P. notatus</i> MR	122	-----
<i>M. musculus</i> MR	893	RTNYIKELRRTVIAENENNTGONWQRFYQLTKLDSMHDLVGLLQICFYTFNKSALKEFFMLVEIINDQFEVTTGM
<i>L. erinacea</i> GR	229	RTNYIKELGKAIVKKEENSSQNWQRFYQLTKLDSMHDLVGLLQICFYTFNKSALKEFFMLVEIINDQFEVTTGM
<i>N. pulcher</i> GR2	715	RMNYIKELGKAIVKKEENSSQNWQRFYQLTKLDSMHDLVGLLQICFYTFNKSALKEFFMLVEIINDQFEVTTGM
<i>N. pulcher</i> GR1	689	RMNYIKELGKAIVKKEENSSQNWQRFYQLTKLDSMHDLVGLLQICFYTFNKSALKEFFMLVEIINDQFEVTTGM
<i>P. notatus</i> GR	215	RMNYIKELGKAIVKKEENSSQNWQRFYQLTKLDSMHDLVGLLQICFYTFNKSALKEFFMLVEIINDQFEVTTGM
<i>M. musculus</i> GR	705	RMNYIKELGKAIVKKEENSSQNWQRFYQLTKLDSMHDLVGLLQICFYTFNKSALKEFFMLVEIINDQFEVTTGM
<i>L. erinacea</i> MR	394	TKSLLEFHK
<i>N. pulcher</i> MR	989	TKSLLEFHK
<i>P. notatus</i> MR	122	-----
<i>M. musculus</i> MR	973	AKSLLEFHK
<i>L. erinacea</i> GR	309	AKSLLEFHK
<i>N. pulcher</i> GR2	795	VKSLLEFHK
<i>N. pulcher</i> GR1	769	VKSLLEFHK
<i>P. notatus</i> GR	294	VKSLLEFHK
<i>M. musculus</i> GR	785	IKSLLEFHK

* C18(Aldosterone) vs. C17(Cortisol), ° - MR/GR unique residues

+ Residues unique to teleost derived GR

GR related earmarks on bottom of each row, and MR earmarks on top

1—C3=O 2—C20=O 3—C21/C11-OH 4—C18=O 5—C17-OH

Table 4.1: Sequence information for LBDs used in the design of the fusion CR construct.

Genbank ID	LBD	canonical domain (aa)	LBD size (aa)	size of GAL4 fusion (kDa)
EF661650	npMR	629 - 993	365	59.4
EF661651	npGR1	471 - 779	309	53.8
EF661652	npGR2	495 - 802	308	53.6
EF092836	pnGR	476 - 779	302	52.9
NM 008173	mmGR	500 - 792	293	51.7
NM 001083906	mmMR	661 - 980	320	54.7

LBD cassettes were then inserted into the pCMV5 mammalian expression vector. This DNA was prepared using Qiagen Maxi prep kits and used in transfections for the western blots and trans-activation assays performed on each fusion product. Refer to Table 4.1 for fusion size and Genbank Accession numbers and Figure 4.2 for details of fusion design.

For the luciferase reporter (pGL3-Luc vector, see Fig. 4.2), a cassette containing five repeating GAL4 response elements (CGGAGTACTGTCCTCCG) upstream of a minimal HSP70 promoter (isolated from a pUAST vector) was ligated into a pGL3-luc vector 100 bp upstream of the luciferase coding region.

Expression and Western blot analyses

The methods used are similar to those reported by Kim et al. (2006) (16). Briefly, 293T HEK cells were grown in DMEM with high glucose (Invitrogen, Carlsbad, CA) containing 10% FBS. The cells were plated in six-well plates and grown to approximately 70% confluence. Each well received 1 µg of pCMV5-GAL4-LBD fusion plasmid DNA (cichlid MR, GR1 and GR2; midshipman GR; mouse MR and GR) for transfection using Genejuice transfection reagent at 3 µl/ µg of DNA (Novagen). Eighteen hours after transfection, the cells were collected and following freeze-thaw in liquid nitrogen, cell extracts were harvested in 1x lysis buffer (Promega, Madison, WI). Cell extracts were mixed 1:1 with SDS loading solution, boiled at 95°C for 5 min, and frozen at -80°C. Expression was determined by polyacrylamide-SDS gel electrophoresis and Western blotting using the FLAG M2 antibody (Sigma) and an ECL chemiluminescent detection system (GE Healthcare/Amersham, Piscataway, NJ).

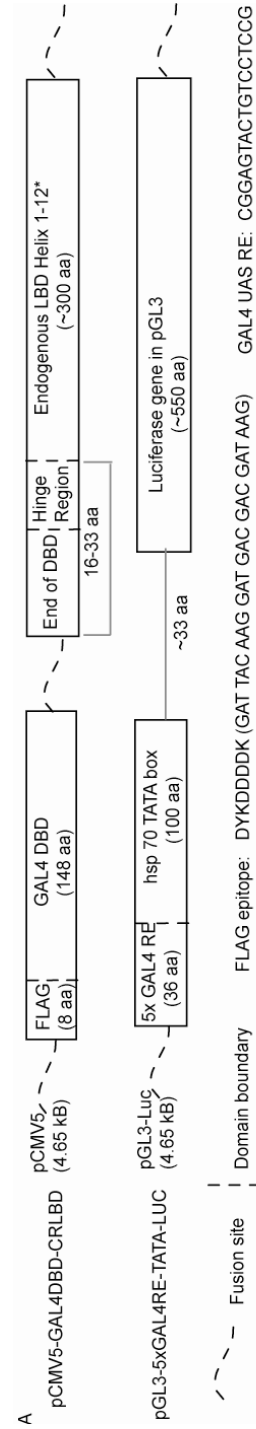


Figure 4.2: The diagram outlines the method for constructing the LBD-GAL4 fusion proteins, including the relative sizes of each specific piece cloned. A dashed-wavy line indicates a fusion point and a dashed-vertical line indicates a domain boundary. The construct on top is the pCMV5-GAL4 DBD-LBD fusion product and the construct on bottom is the pGL3-UAS (5XGAL4-TATA)-Luciferase reporter product. Each fusion piece is listed with its canonical location, LBD transcript size (amino acid), and fusion-product size (kDa).

Transactivation Assays

The methods used are similar to those reported by Kim et al. (2006) (16). Briefly, HeLa cells were grown in DME/F-12 (Invitrogen, Carlsbad, CA) containing 10% CDCS. The cells were plated in six-well plates and grown to approximately 70% confluence. The cells in each well were transfected with the following combinations of plasmid DNAs: (1) 400 ng of the appropriate pCMV5-GAL4-LBD vector for the expression of the gene-fusion products (mouse vs. cichlid, GR vs. MR), or 400ng of a control pCMV-GAL vector with no LBD, and (2) 500 ng of a GAL4 responsive pGL3 luciferase reporter construct containing five GAL4 response elements (pGL3-5xGAL4RE). Twelve hours after transfection, the cells were treated with vehicle (ethanol) or 0.01 μ M of hormone for an additional 18h (all steroids from Sigma, St. Louis, MO): aldosterone, dexamethasone, hydrocortisone (cortisol), 11-deoxycortisol, cortisone and 11-ketotestosterone (11-KT) which is the major non-aromatizable androgen found in teleosts, comparable to dihydrotestosterone (DHT) in mammals, although some mammals also have low levels of 11-KT (1, 17). Both cortisone and 11-deoxycortisol were chosen to aid in distinguishing cortisol specificity because of their place in the steroid biosynthetic pathway: cortisone is a direct by-product of cortisol metabolism and 11-deoxycortisol is a direct precursor to cortisol (1). Dexamethasone was chosen because of the well-established high binding affinity of this synthetic glucocorticoid to the mammalian GR (12, 18, 19). The androgen 11-KT was chosen to examine whether the isolated LBDs show generalized binding to C3-keto steroids (see Bentley, 1998 for structure). The above combination of steroids enabled the assessment of the predicted discriminatory basis for each LBD fusion product.

Luciferase activity in cell extracts was measured using a mix of 50 μ l luciferin (1:1 with water) and 50 μ l extract. The raw luciferase values for each individual fusion

product were then normalized to the values for the vehicle (ethanol) treatment specific for that LBD. To ensure reproducibility, each assay was run in duplicate, and each experiment was performed at least four times.

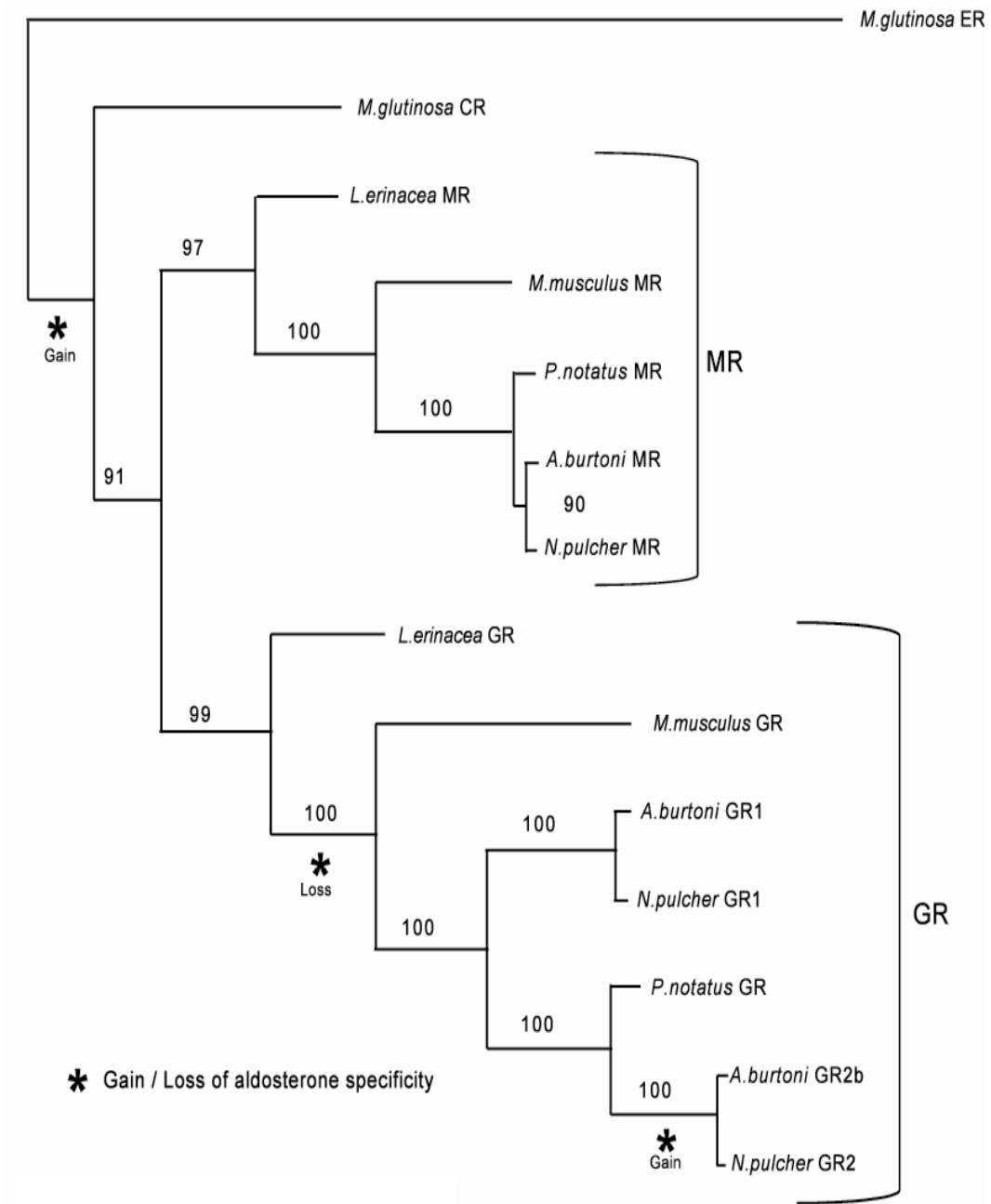
Statistics

Statistical comparisons were performed using ANOVA analysis and post-hoc Tukey HSD with JMP 7.0 software. All normalized values were tested for significance over baseline (vehicle treatment = 1).

Phylogenetic Analysis

Phylogeny was estimated using MrBayes 3.1.2 (20) run at the facilities of the Computational Biology Service Unit of Cornell University. In addition to the sequences used in this study, the following sequences were added for phylogenetic resolution (full length sequences were used where available): *Myxine glutinosa* (hagfish) estrogen receptor (ER) (Genbank accession No. EU439936.1), *Myxine glutinosa* CR (Genbank accession No. DQ382336.1), *Leucoraja erinacea* (skate) MR (Genbank accession No. DQ382339.1), *Leucoraja erinacea* GR (Genbank accession No. DQ382338.1), *Astatotilapia burtoni* (Burton's cichlid) MR (Genbank accession No. AF263741), *Astatotilapia burtoni* GR1 (Genbank accession No. AF263738.1), *Astatotilapia burtoni* GR2b (Genbank accession No. AF263740), *Porichthys notatus* (plainfin midshipman) MR (Genbank accession No. EU926160.1). A second isoform, GR2a, for *A. burtoni* was not included in the alignment because it shows a 100% sequence identify with the partial sequence of *A. burtoni* GR2b used in the analysis (see (10). A phylogram was created using the sequences for the fusion LBDs along with complete (where available) sequence of other vertebrates (hagfish as the outgroup (9) and skate; see Fig. 4.3) to aid in functional interpretations. A general time reversible

Figure 4.3: A phylogram of the relationship between the protein sequences of the GRs and MRs used in the alignment in Figure 3 that includes the LBDs subjected to trans-activational assays in the current study. An additional cichlid species (*A. burtoni*) used in earlier transactivational studies (9) has also been included. Branch confidence is represented in relative percentage next to the branch. A star indicates the gain or loss of aldosterone sensitivity according to the cladistic placement and reported specificity. As shown, the stars indicate that the aldosterone sensitivity of the ancestral corticosteroid receptor (represented here by the hagfish, *M. glutinosa*) was retained by the MR and GR of cartilaginous fishes, (represented here by the skate *L. erinacea*), but was subsequently lost by the GR of tetrapods (represented here by a mammal, *M. musculus*) and teleost fish (represented here by the cichlids *A. burtoni* and *N. pulcher*, and the midshipman *P. notatus*). Aldosterone specificity secondarily evolved among teleosts with a duplicated GR (represented here by cichlid GR2).



(GTR) model with invariable sites and a gamma distribution for variable rate sites (GTR+I+G) was applied. Four Markov chains of 1,000,000 generations were run sampling every 100th tree and a burn-in of 25% was applied to remove all trees generated in the first quarter of the analysis. From the remaining trees, a majority rule consensus tree was generated along with posterior probabilities. The analysis was replicated to confirm both the consensus tree and the posterior probabilities. To test alternative hypotheses, the resulting consensus tree was edited using Mesquite 2.5 (21) and hypothetical trees with alternative topologies were compared with the consensus tree in PAUP*4.0 (22) using a Shimodaira-Hasegawa (SH) likelihood test with 1,000 bootstrap replicates and full optimization.

Results

Cloning, alignment and phylogeny of corticosteroid receptors

After the initial cloning of each LBD, the resulting sequences were subject to multiple alignments to ensure both sequence integrity and species identity (see Fig. 4.1 for sequence alignments and Table 4.2 for sequence identity). The cloned sequences of the corticosteroid receptors included the entire coding region for the LBD and a partial DBD sequence at the amino ends. From this alignment, a phylogenetic analysis was performed to estimate the relationship between the vertebrate corticosteroid receptors. The resulting phylogram (see Fig. 4.3) included posterior probabilities that were expressed as percentages above each branch. A polytomy between the hagfish estrogen receptor, the outgroup, and the hagfish corticosteroid receptor was the only modification to the tree; an SH test confirmed no significant difference between the two trees ($p>0.8$). As suggested previously (22, 29, 30), the phylogram revealed that the estrogen receptor is ancestral to any corticosteroid-related protein and that the hagfish corticosteroid receptor is ancestral to any GR versus MR divergence.

Table 4.2: Sequence identity table for the genes used in the phylogram and transactivation studies.

	<i>M.glutinosa</i> ER	<i>M.glutinosa</i> CR	<i>M.musculus</i> MR	<i>N.pulcher</i> MR	<i>A.burtoni</i> MR	<i>P.notatus</i> MR	<i>Lerinacea</i> MR	<i>Lerinacea</i> GR	<i>A.burtoni</i> GR2	<i>N.pulcher</i> GR2	<i>M.musculus</i> GR	<i>A.burtoni</i> GR1	<i>N.pulcher</i> GR1	<i>P.notatus</i> GR
<i>M.glutinosa</i> ER	100	23	26	22	24	26	26	23	28	24	26	27	22	20
<i>M.glutinosa</i> CR	23	100	55	56	58	61	56	56	54	50	57	56	51	50
<i>M.musculus</i> MR	26	55	100	71	73	75	70	63	61	58	60	62	56	55
<i>N.pulcher</i> MR	22	56	71	100	98	96	64	60	56	56	56	56	55	53
<i>A.burtoni</i> MR	24	58	73	98	100	95	66	60	60	56	58	60	55	53
<i>P.notatus</i> MR	26	61	75	96	95	100	69	65	54	54	52	51	51	52
<i>Lerinacea</i> MR	26	56	70	64	66	69	100	61	62	60	60	63	58	57
<i>Lerinacea</i> GR	23	56	63	60	60	65	61	100	64	64	63	65	64	62
<i>A.burtoni</i> GR2	28	54	61	56	60	54	62	64	100	99	74	82	80	86
<i>N.pulcher</i> GR2	24	50	58	56	56	54	60	64	99	100	70	80	80	86
<i>M.musculus</i> GR	26	57	60	56	58	52	60	63	74	70	100	74	69	68
<i>A.burtoni</i> GR1	27	56	62	56	60	51	63	65	82	80	74	100	99	79
<i>N.pulcher</i> GR1	22	51	56	55	55	51	58	64	80	80	69	99	100	79
<i>P.notatus</i> GR	20	50	55	53	53	52	57	62	86	86	68	79	79	100

Additionally, the analysis suggested multiple GR-related modification events over the course of vertebrate evolution that resulted in duplicate GR genes among fish. An initial duplication event among teleosts gave rise to two GRs, GR1 and GR, while a later duplication gave rise to the GR2.

Ligand responsiveness of the receptor LBDs

The activity of some corticosteroid receptors has been previously assayed using different cell based reporter assays (9, 10, 23, 24). To determine the ligand responsiveness of each of the cloned receptor LBDs, we used a cell based reporter gene assay to test the activity of corticosteroid receptors from both a mammal (mouse) and two distantly related groups of teleost fish (the cichlid *N. pulcher*, the midshipman *P. notatus*). The LBDs were attached to a GAL4-DBD and these cassettes were then inserted into the pCMV5 mammalian expression vector. The hormone-induced activation of these fusion constructs was monitored using a GAL4 response element that is “off” unless in the presence of GAL4-DBD activation. This allowed the use of a reporter with low basal promoter activity (the 5X GAL4-TATA sequence from the pUAST construct commonly used in *D. melanogaster*; see methods) that has been used extensively for monitoring binary gene expression in many systems and is similar to enhancers of multicellular eukaryotes (25). This system represents a cautious approach to interspecific comparisons because the DBD of different species may interact to a different degree with endogenous promoters (26), an issue that we avoided by using a heterologous DBD.

After designing the fusion constructs, we wanted to test the hypothesis that corticosteroid receptor proteins that are proposed to be homologous among vertebrates respond with the same specificity to a panel of hormone treatments. We first confirmed expression of the full-length LBD-GAL4 fusion products from the plasmid

constructs by Western blotting (Fig. 4.4). We then measured the luciferase activity produced by each fusion construct in response to hormone treatments including natural and synthetic glucocorticoids, a mineralocorticoid, and an androgen. Normalized values were expressed as fold change over vehicle (ethanol) treatment (Fig. 4.5). Significant differences ($p \leq 0.05$, indicated by asterisks above bars in Fig. 4.5) in ligand specificity were observed between the GR and MR LBDs of all species examined. The mouse GR showed significantly higher than a four-fold activation over vehicle baseline values for the glucocorticoid-specific ligands, cortisol (Reichstein's compound M, Fig. 4.5) and dexamethasone (Dex, Fig. 4.5), with no response to any of the other steroids including aldosterone (Aldo, Fig. 4.5). The mouse MR showed a significant two- to three-fold activation over vehicle baseline for cortisol and dexamethasone but responded with a significant three- to four-fold activation for the mineralocorticoid aldosterone. These results for the mouse GR and MR validated the specificity and sensitivity of this reporter system (2). Similarly, the putative cichlid MR showed above a two-fold activation for cortisol and dexamethasone, and above a three-fold activation for aldosterone. This result supported the hypothesis that the cichlid MR may be representative of the teleost MR in general as its ligand specificity mimics that of the mouse MR (see 9).

Comparable to mouse GR, cichlid GR1 and the midshipman GR showed, respectively, above a three- and two-fold activation to cortisol and dexamethasone. However, neither the cichlid GR1 nor the midshipman GR responded to aldosterone or any of the other steroids that were tested. The cichlid GR2 showed about a two-fold activation for cortisol and dexamethasone, but unlike other GRs, it also showed a significant two-fold activation in response to aldosterone. In addition, neither the glucocorticoid cortisone (Reichstein's compound F, Fig. 4.5) nor the non-aromatizable androgen 11-KT induced activation in any of the LBDs. While 11-deoxycortisol

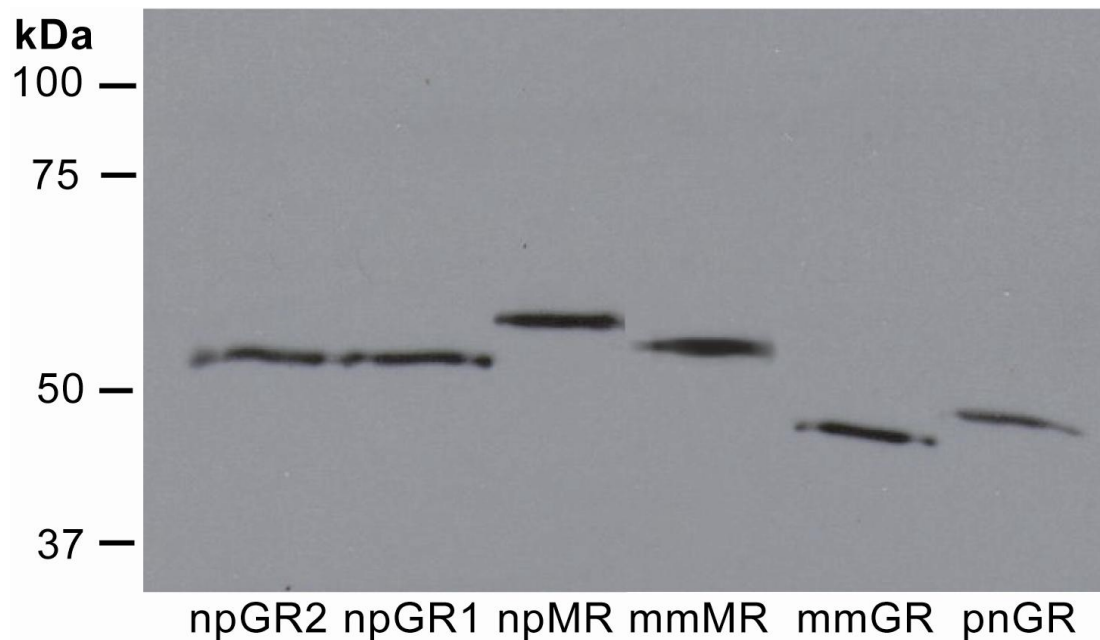
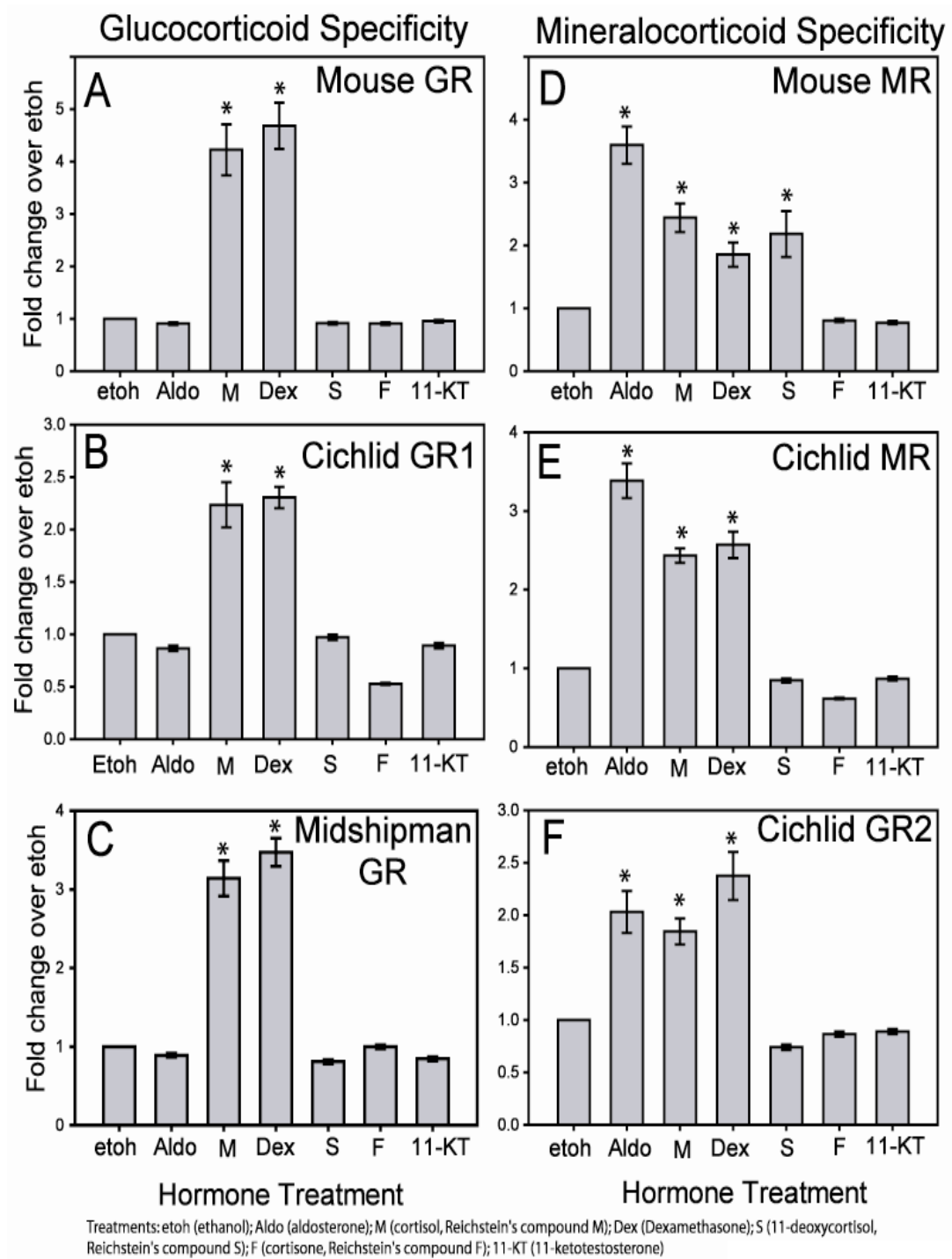


Figure 4.4: Full-length fusion constructs were transfected using 293T cells and cell extracts were run by SDS-PAGE, treated with primary FLAG antibody and secondary Donkey anti-mouse HRP, and imaged on xray film using ECL exposed for 1.5 mins. The resulting image showed proteins of the expected size (scale to left) and verified the expression of the full length fusion construct. Mouse GR – *M. musculus* (mm)GR; Mouse MR – mmMR; Cichlid GR1 – *N. pulcher* (np)GR1; Cichlid MR – npMR; Cichlid GR2 – npGR2; midshipman GR – *P. notatus* (pn)GR.

Figure 4.5: The graphs show the relative fold-activation over ethanol baseline for each fusion-construct to steroid treatments (ethanol - etoh; Aldosterone – Aldo; Cortisol – compound F; Dexamethasone – DEX; 11-deoxycortisol – compound S; Cortisone – compound E; 11-ketotestosterone-11-KT). The mouse GR and MR show the expected specificity and indicate the validity of the assay. The duplicate GR of cichlid (GR2) showed an aldosterone response and represents a GR that has an ancestral (MR-like) phenotype.



(Reichstein's compound S, Fig. 4.5) induced a significant amount of mouse MR activity (two-fold above baseline), no comparable activity was observed for any other LBD.

Sequence analysis

Since residues outside of the ligand pocket have been suggested to provide additional interaction with ligands (27-29), we analyzed the LBD sequences used in this study to identify potential residue alterations in these regions that may aid in the difference in aldosterone sensitivity between duplicated teleost GRs. Several residues stand out in the LBD of GRs compared to MRs. For example, the following residues in the mouse MR are specific to all MRs examined in the alignment: Thr862, Asn895, Gly912, Ser812, Ala770, Lys950, Arg901, Asp895 (see Fig. 4.1). Conversely, the equivalent GR residues are divergent from those in the MR, but conserved within GRs including teleost GR/GR1 and GR2. Hence, such divergence is likely to contribute to the ligand specificity of mammalian GR versus MR, but not GR2's aldosterone specificity. Similarly, a series of shifts seem to have occurred between mammalian and teleost GR homologues; however, these residues are common within all teleost GRs and therefore do not represent a divergence between teleost GR paralogues. Thus, these alterations are also not likely to contribute significantly to GR2's aldosterone-sensitive phenotype, although they may contribute to differences in ligand activation between mammalian and teleost GRs.

Lastly, our analysis reveals a final set of residue changes between the duplicated GR paralogues of teleosts that may contribute to the aldosterone specificity observed for GR2 (Fig. 4.1). For example, residues such as Leu763/ Ser764 (LS) in the mouse MR are combinations found only in those corticosteroid receptors that are sensitive to aldosterone (cichlid MR, skate MR, skate GR, mouse MR, and

midshipman MR; see Fig. 4.1). In mouse GR, as well as midshipman GR and cichlid GR1, these residues have shifted to Met575/ Thr576 (MT). Hence, these shifts occur in a manner that is consistent for all of the vertebrate MRs versus GRs studied here, except for cichlid GR2 that shows aldosterone sensitivity. The best description of GR2's residues at these locations is that they are similar to both GRs and MRs. Thus, GR2 is more similar to the common vertebrate GR in the first position (indicated by a Methionine), but more similar to the common vertebrate MR in the following position (indicated by a Serine – see Fig. 4.1). Although Thr576 in mouse GR may be considered a closely related substitution for Ser764 in mouse MR, the alignment in Figure 4.1 indicates that this residue is located adjacent to residues critical for binding to the C3/C18 carbonyl and C21/C11 hydroxyl groups of the aldosterone steroid backbone (see Fig. 4.6). Therefore, changes around these functional residues that are outside but adjacent to binding sites may influence ligand preference of either a GR (e.g., GR2) or an MR for aldosterone.

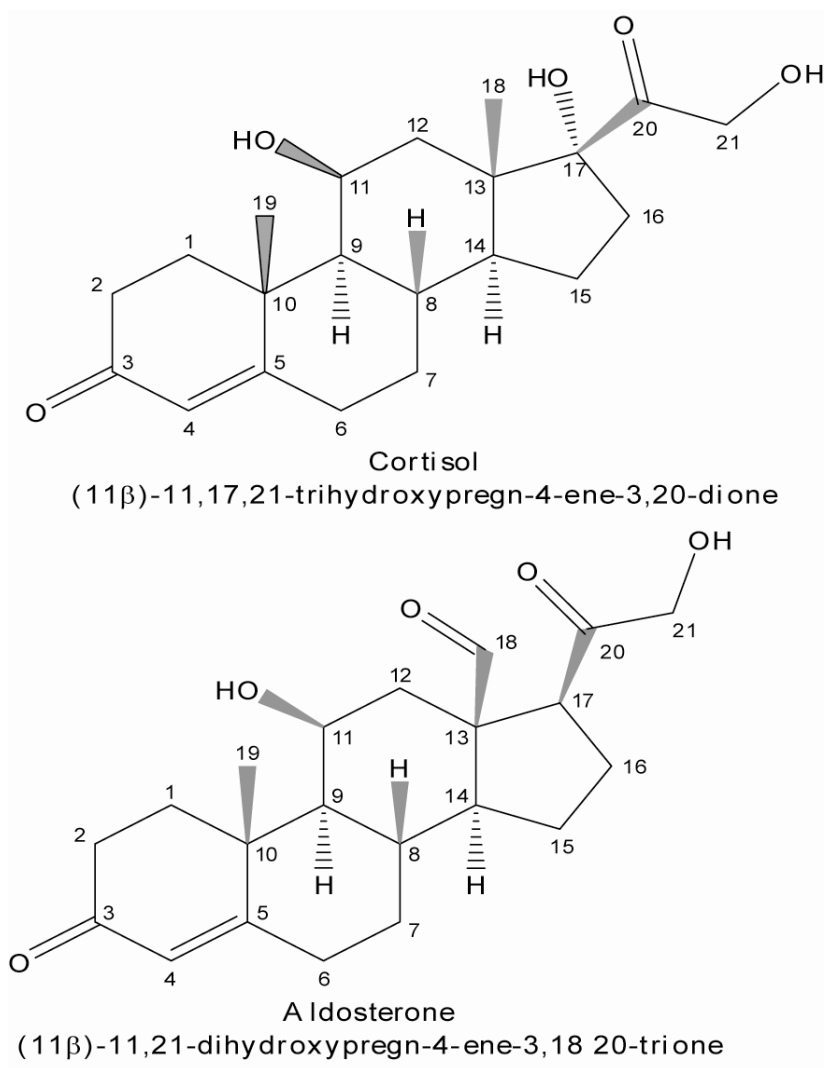


Figure 4.6: The steroids cortisol and aldosterone are shown with the IUPAC name and carbon-atom numbering appropriately labeled. Note that aldosterone differs from cortisol at the carbon 17 and carbon 18 positions. Cortisol and aldosterone have a C17 hydroxyl group and a C18 carbonyl group, respectively, as indicated in their chemical name and on their structures.

Discussion

We demonstrate that close evolutionary relationships in the protein sequences for corticosteroid receptors can fall short in predicting profiles of ligand-induced transcriptional activation. For example, teleost fish receptors with a high sequence identity to mammalian GRs are activated like mammalian GRs by cortisol, but not by aldosterone. However, a duplicated teleost GR homologue is activated by both cortisol and aldosterone (also see 9), like mammalian MR and the GR homologue of a more primitive cartilaginous fish (9). Additional analysis suggests that key residue changes between the teleost GR paralogues influence the receptor's ligand sensitivity. Together with a phylogenetic analysis of the protein sequences of corticosteroid receptors, the results show that sequence identity is not always predictive of a steroid receptor's ligand specificity across species.

Evolution of divergent GR sequences and ligand specificity

It has been suggested from crystal structures of the mammalian GR and MR (based on single residue mutations) (12, 30) that since the binding sites with glucocorticoids are conserved between GR and MR, the differences in specificity are due to steric hindrance between polar residues within the protein and polar sub-functional groups on the steroid (29). The current methods were used as a surrogate for ligand binding to dissociate it from other components of the canonical receptor, namely the C and F domains. The results presented here show that the LBD alone is capable of ligand discrimination independent of the endogenous AF-1 (C) domain, although its presence has additional modular influences (12). Even under such basal conditions, the isolation of the endogenous LBD revealed a ligand specificity characteristic of the mammalian GR and MR (27). However, within duplicated GRs in teleosts, an aldosterone-sensitive phenotype seems to have been secondarily evolved

by one of the paralogues.

The duplicated GR in cichlid fish, GR2, is most similar in ligand specificity to the GR identified in the more primitive cartilaginous fish, the skate *L.eirnacea* (Fig. 4.3) that shows activation by both glucocorticoids and mineralocorticoids (9) (also see Fig. 4.5). Prior studies suggest that the aldosterone specificity of GR2 is the result of novel function gained via random mutations (24, 31, 32). However, we propose that the aldosterone specificity observed for GR2 was acquired via selective residue mutations. as studies from zebrafish and other teleosts reveal a single GR and MR gene indicating that the presence of GR2 may be the product of an evolutionary event specific to certain teleost species (33). Our combined phylogenetic and functional analyses support the hypothesis that the aldosterone-sensitivity of the GR paralogue (cichlid GR2 in our analysis) is a more recent gain-of-function event subsequent to the initial loss of aldosterone sensitivity by a teleost corticosteroid receptor (GR/ GR1) with high sequence identity to the mammalian GR (Fig. 4.3 and Table 4.2).

The single GR in zebrafish and midshipman shows a sequence identity most similar to cichlid GR2 (7). However, our studies combined with previous ones in another cichlid (10) suggest that the GR duplicates/ paralogues differ in their ligand specificity. Thus, GR1 (also see 23), shows activation by glucocorticoids only (cortisol and dexamethasone) whereas GR2 shows activation by both glucocorticoids and the mineralocorticoid aldosterone. The phylogram in Figure 4.3 indicates that GR2's aldosterone sensitivity is a derived trait that secondarily evolved among some teleost fish. While aldosterone sensitivity is ancestral to both GRs and MRs, given its presence in both the hagfish corticosteroid receptor (22) as well as the skate GR and MR (9), the common GR of tetrapods and teleosts lost aldosterone sensitivity sometime after the initial divergence of the ancestral vertebrate corticosteroid receptor into GR and MR. In some species, the duplicated GRs may have been sub-

functionalized (i.e., served complementary functions, see (34) which may have increased the probability for later transitions to neo-functionalized (i.e., novel, non-complementary functions) states. Conversely, it has been proposed that there was a secondary loss of a duplicated GR in zebrafish due to a lack of sub-functionalization, otherwise its loss would have been quite detrimental (35). Therefore, it may be the case that GR2 was not positively selected for function in all teleost fish. Other studies illustrate the independent evolution of regulatory genes (ion channel genes and others) in teleost fish that have resulted in duplicated sub/neo-functionalized genes as the products of whole genome duplication events (36-42). Therefore, a secondary event of the GR gave rise to a GR2 paralogue with aldosterone sensitivity.

Binding of functional groups to LBD

Steroids consist of a common backbone of four fused carbon rings (A-D) derived from cholesterol, and are distinguished by the functional groups and oxidation state associated with those rings (See Fig. 4.6). Conserved functional groups on the steroid backbone at key positions influence their interaction with the cognate receptors (12). For example, the binding of 3-ketosteroid receptors (Androgen Receptor - AR, Progesterone Receptor - PR, Glucocorticoid Receptor - GR, Mineralocorticoid Receptor - MR) is dependent on the presence of a carbonyl group at the carbon 3 position (C3=O) (18). The C3 group binds to the Gln776 and Arg817 residues in the human MR (corresponding to Gln772 and Arg813 in mouse MR, see black circle 1 in Fig. 4.1) (18, 42). The C20 carbonyl, which is common to both glucocorticoids and mineralocorticoids, binds the residues Cys942 and Thr945 in the human MR (corresponding to Cys938 and Thr941 in mouse MR, see black circle 2 in Fig. 4.1) (28, 29). Also in common to both glucocorticoids and mineralocorticoids are the C21 and C11 hydroxyl groups that bind Asn770 and Thr945 in the human MR

(corresponding to Asn766 and Thr941 in mouse MR, see black circle 3 in Fig. 4.1) (42, 43). Finally, with regard to the functional groups that separate cortisol from aldosterone, the C18 carbonyl in aldosterone binds Asn770 and Cys942 in the human MR (corresponding to Asn766 and Cys938 in the mouse MR, see black circle 4 in Fig. 4.1) (29, 44), while the C17 hydroxyl in cortisol binds Glu642 in human GR and Leu848 in human MR (corresponding to Leu844 in mouse MR, see black circle 5 in Fig. 4.1) (12, 42). The change in polarity between the hydroxyl and carbonyl groups at the border of the C and D ring (C17 hydroxyl in cortisol versus C18 carbonyl in aldosterone) of the steroid backbone may influence the interaction of functional groups at these locations with the GR and MR (see introduction).

The removal of a hydroxyl group at C17 would shift, although maybe only slightly, the dipole of the electrostatic polarity toward the backbone and away from the functional group, thereby decreasing the probability of polar interactions between this functional group and Glu642 in human GR (L848 in human MR has already been proposed to maintain such action, see above). The addition of a carbonyl group at the C18 position on the steroid backbone may also shift the dipole slightly away from the backbone and toward the functional group increasing the probability of polar interactions between this functional group and Asn770/Cys942 in human MR. However, it should be noted that corticosterone, which lacks the C17 hydroxyl and C18 carbonyl, binds the human and mouse GR well (2). In the human MR, the binding of the C18/C20 carbonyl and the C11/C21 hydroxyl stabilizes AF2 and its interaction with Ser767 and Glu955 (Ser763 and Glu951 in mouse MR) (29). Although the above residues are members of the ligand pocket and influence binding, it has also been shown that residues outside of the pocket can influence ligand binding. For example, the region of residues 820-844 in the human MR are important for aldosterone binding and likely sensitivity as it is quite variable between human GR and MR (28), but this

region is not within the ligand pocket of the confirmed protein. In addition, the residue Glu962 in the human GR maintains a different orientation in the ligand-bound confirmation than the equivalent residue in the human MR, Glu755 (26).

Further teleost GR and MR considerations

Our analysis suggests that the residue Serine (764) in mouse MR, equivalent to Threonine (576) in mouse GR, may be predictive of aldosterone specificity across vertebrates (where Serine is common to aldosterone sensitive CRs – see Fig. 4.1 and Fig. 4.5). Accordingly, our previous analysis of an alignment of vertebrate GRs (43) predicts that like the GR2 in cichlids the following duplicated GRs may be sensitive to aldosterone: *Takifugu rubripes* GR1 (Ensembl identifier: ENSTRUG00000006399), *Oryzias latipes* GR1 (Ensembl identifier: ENSORLG00000001565), *Tetraodon nigroviridis* GR2 (Ensembl identifier: ENSTNIG000000018209). These proteins may represent duplicated GRs that have been modified for aldosterone specificity. Similarly, our analysis of vertebrate MRs (4) may predict an aldosterone insensitivity in the MRs of zebrafish (*Danio rerio*, Genbank accession No. EF567113) and common carp (*Cyprinus carpio*, Genbank accession No. AJ783704.2), as indicated by a Threonine at the residue that aligns with Serine764 in the mouse MR. These proteins may represent MRs that adapted for cortisol binding. Therefore, opposite trends for either a gain or a loss of aldosterone specificity, may occur, respectively, in MRs and GRs. In these cases, the functional classification of corticosteroid receptor proteins based on sequence identity again may not accurately predict their respective ligand specificities. However, a higher degree of sequence divergence ($\leq 85\%$ versus $\geq 90\%$) in concert with the above residue shift, may indicate divergent phenotypes within GRs and MRs. Unfortunately, trans-activation data is not available from enough species to further resolve this hypothesis, but such data can best be acquired

using the same assay as done here and to test this hypothesis.

As previously mentioned, the physiological significance of divergent corticosteroid receptors among teleosts has remained somewhat of an enigma given the apparent absence of biologically relevant levels of circulating aldosterone. We tested the specificity of these receptors for cortisol (F) as well as intermediates/precursors (11-deoxycortisol - S) and metabolites (cortisone, E) of cortisol synthesis (Fig. 4.5). The specificity revealed for cortisol and not other synthetically related hormones is remarkable. However, there are a number of other candidate ligands that might help define receptor specificity and functionality among teleosts. For example, the addition of other hormones that are synthetically related to aldosterone synthesis (precursors such as 11-DOC and corticosterone) may aid to further detail the ancestral relevance of such a sensitivity. In addition, dose-response assays combined with site-directed mutagenesis studies aimed at the residue shifts commented on here (see Fig 4.1) may also provide more determinate information regarding comparisons between divergent corticosteroid receptor proteins and species-specific differences in sensitivity. Such a comparative study could yield novel insights into the potential regulatory roles different affinities of homologous corticosteroid receptors for the same ligand may have between species (i. e., cortisol vs. corticosterone in mouse and humans, cortisol vs. aldosterone in mammals and teleosts). Furthermore, aldosterone sensitive corticosteroid receptors in teleosts may be more indicative of a receptor that has high affinity for glucocorticoids, as is the case for the mammalian MR which shows significantly greater affinity for cortisol than the GR. Dose-response investigations using methods similar to those described here could also provide fundamental comparative data that may prove vital to the accurate functional interpretations of such differences in affinity.

Evolution of ligand divergence

Since the ancestral corticosteroid receptor (hagfish and lamprey) is sensitive to both cortisol and aldosterone (9, 31), the evolutionary events that produced the corticosteroid receptor targeted both C11/C21 hydroxyl and C18 carbonyl functional groups. Comparing the functional group differences between cortisol and aldosterone (C17 hydroxy versus C18 carbonyl, respectively) can be quite informative on both functional and evolutionary levels of analysis. We have already discussed the loss of aldosterone (C18 carbonyl) specificity in the GR and how this change influenced the evolution of vertebrate corticosteroid receptors. However, similar mechanisms are likely to be widespread within the steroid receptor gene family. For example, the estrogen receptor (specific for 17 β -estradiol which has C3 and C17 hydroxyl functional groups, see Bentley 1998) has been proposed to be the ancestral steroid receptor whose initial duplication led to the divergence of all other steroid receptor groups (26). A single C3-ketosteroid receptor (specific for a C3 carbonyl group) was a product of such an event and the further duplication of this single C3-ketosteroid receptor led to androgen receptor, progesterone receptor, GR, and MR. Therefore, the initial driving force for divergence in steroid receptors was the preference for a C3 carbonyl (C3-ketosteroid receptor) versus a C3 hydroxyl group (estrogen receptor). Both the androgen and progesterone receptors have been proposed to evolve from the ancestral C3-ketosteroid receptor prior to the appearance of corticosteroid receptors (26, 27). In addition, the binding profile of the progesterone receptor more closely resembles that of corticosteroid receptors (19, 27, 30), which provides support for the divergence of CRs after the progesterone receptor. However, at the time of the divergence of CR from other C3-ketosteroid receptors the molecular machinery necessary for aldosterone production was not available.

Two essential enzymes would be needed to secure a functional role for

aldosterone. The first enzyme (aldosterone synthase) is required for aldosterone synthesis and the earliest report of its expression is in early tetrapods (5). The second enzyme (11 β HSD) has two isoforms in tetrapods and mammals and is required for the preferential synthesis of aldosterone over cortisol (5). While one isoform of the enzyme has been reported in teleost fish (11 β HSD2), it is the presence of both enzymes that establishes an aldosterone-induced versus a cortisol-induced MR activation, as cortisol is generally present at much higher levels (44-47). It is clear that the diversification of the GR and MR, mainly for the specificity for C17 hydroxyl and C18 carbonyl, was mainly on the part of the GR and its loss for aldosterone specificity. The inherent conclusion to such an analysis is that GR and MR continued the ancestral trend of steroid receptor diversification toward interactions with specific functional groups on the steroid backbone. While much of the above discussion focused on the divergence of steroid receptors for specific functional groups of intermediates from estrogen synthesis, a C18 carbonyl functional group is an aspect that is unique only to aldosterone and no other steroids. In this case it may be the corticosteroid receptor driving the evolution of the ligand (aldosterone) whereas traditionally it has been thought that the ligands (downstream products of estrogen metabolism) have driven the divergence/duplication of the receptors. This critical bidirectional/ co-evolutionary mechanism is likely to be fundamental to the gain or loss of aldosterone sensitivity among corticosteroid receptors (e.g., cichlid GR2 and the initial MR-GR split) over the course of evolution. Small single-residue differences between homologous vertebrate proteins that parallel differences in function, such as between GR and GR2 with aldosterone, may not be an event unique to GRs or teleost fish. In addition to differences in aldosterone sensitivity and regulation between mice and primates, there are also well established differences in the specificity of CRs for cortisol versus corticosterone (48-50). In support of such differences, our results

indicate that the mouse MR has a sensitivity for 11-deoxycortisol, a precursor to cortisol synthesis, while corticosterone is the main glucocorticoid in mice. Therefore, recent evolutionary events within both GR and MR proteins may have relevance to mammals and indicate that small residue changes resulting in dynamic functional consequences may not just occur in fish, but in tetrapods as well.

Conclusions

The current report used a single luciferase reporter assay to characterize the transcriptional activation of corticosteroid receptors across the two most widely divergent vertebrate groups (mammals and teleost fish). The results showed teleost receptors with sensitivity to either cortisol or cortisol and aldosterone like, respectively, the classic mammalian GR and MR. The teleost receptor that is sensitive to aldosterone contains residues that may favor MR-like polar interactions with aldosterone. Teleosts have duplicated receptors closest in sequence identity to the mammalian GR. Phylogenetic analyses showed that the aldosterone-sensitive GR paralogue (GR2) gained such specificity after its initial loss at the time of the MR/GR divergence with the origin of bony fishes. These molecular events reflect the dynamic and complex regulation linked to the divergence of corticosteroid receptors across vertebrates. Such investigations will continue to provide a platform for discussion of comparative genomics and endocrinology.

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